Estimation of Dental Plaque Levels and Gingival Inflammation Using a Simple Oral Rinse Technique

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A SIMPLE, NON-INVASIVE TEST (the Oratest) has recently been proposed, which provides an estimate of oral microbial levels based on the rate of oxygen depletion in expectorated milk samples. Following 30 seconds of vigorous rinsing with sterilized milk, 3 ml of the expectorate is added to a test tube containing the redox indicator, methylene blue, and the time required for a color change from blue (i.e., aerobic conditions) to white (anaerobic conditions) at the bottom of the test tube is recorded. In the present study, Oratest scores were compared to clinical parameters (Plaque Index [PI] and Gingival Index [GI]) in a group of 49 volunteers. Significant correlations were found between the logarithm of Oratest results and PI ($r = -0.58$; $P = 0.001$) as well as GI ($r = -0.66$; $P = 0.001$). The data indicate that the Oratest provides a reliable estimate of gingival inflammation, thus extending the previously reported strong correlations between Oratest scores and microbial counts. The data suggest that the Oratest may have potential as a clinical and research tool. *J Periodontol 1990; 61:339–342.*

Key Words: Plaque index; gingival index; Oratest; mouth/microbiology; gingivitis.

A large body of evidence supports the hypothesis that microorganisms present in dental plaque constitute the primary etiological factor in periodontal diseases.[1–3] It has similarly been established that dental plaque accumulation induces and promotes gingivitis.[4] Prior to the 1970s, periodontal diseases were ascribed to the overall increase in microorganisms, a concept referred to as the "non-specific plaque hypothesis."[5] More recently, specific groups of bacteria were found to be associated with particular periodontal ailments, leading to the "specific plaque hypothesis."[5] In 1985, Moore et al.[6] reported that periodontal diseases are associated with elevated levels of at least 57 bacterial species. The observation that many suspected periodontal pathogens require or prefer anaerobic growth conditions has led to speculation that oxygen depletion by aerobic plaque microorganisms is a prerequisite for the development and progression of periodontal diseases.[7]

Recently, Rosenberg and coworkers[7] reported a simple, non-invasive technique (the Oratest‡) which estimates oral microbial levels, based on the rate of oxygen depletion in expectorated milk samples. The test is performed by rinsing the mouth with sterilized milk. A sample of the expectorate is then added to a test tube containing the redox indicator, methylene blue. The time required for anaerobic conditions to be attained at the bottom of the tube, as indicated by a blue-to-white color change, is recorded. In a previous investigation, strong correlations were observed between Oratest scores and counts of aerobic and aerotolerant microorganisms.[7] The present study was undertaken to compare Oratest scores with commonly used techniques for clinical evaluation of plaque levels and gingival inflammation.

MATERIALS AND METHODS

Forty-nine volunteers (mean age 42.7 years) participated in the study. The plaque level of each volunteer was scored using the Plaque Index (PI) described by Quigley and Hein:[8] 0 = no visible plaque; 1 = isolated flecks of stain at gingival margin; 2 = continuous definite line of plaque at gingival margin; 3 = gingival third surface covered by plaque; 4 = 2/3 of surface covered by plaque; and 5 = more than 2/3 of surface covered by plaque. The severity of gingivitis was assessed using the GI system of Löe and Silness:[9] 0 = clinically normal, pink color, no edema or altered contour; 1 = mild inflammation, slight change in color (erythema) and little change in texture; 2 = moderate inflammation, moderate glazing, redness, edema, and enlargement, bleeding on pressure with a blunt instrument; and 3 = severe inflammation, marked redness and enlargement, tendency for spontaneous bleeding.

The Oratest has been previously described.[7] Briefly, vol-

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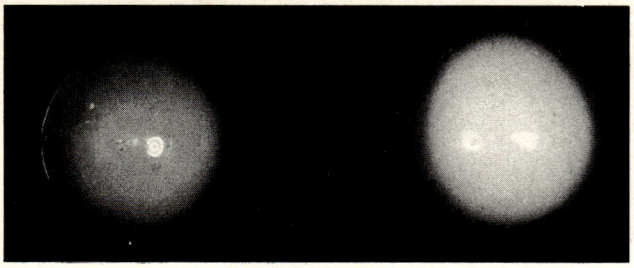

Figure 1: The Oratest. The bottom of the test tubes, prior to (left) and following (right) the blue-to-white color change.

Table 1. Relationship between Oratest Results* and Plaque Index

Population	Number	Correlation Coefficient	Significance
All volunteers	49	r = −0.58	$P = 0.001$
Did not eat or drink†	30	r = −0.64	$P = 0.001$
Did eat or drink†	19	r = −0.48	$P = 0.019$

*Logarithm of time (minutes) required for blue-to-white color change at bottom of test tube.
†90 minutes prior to test.

unteers rinsed their mouths vigorously with 10 ml of ultra-high-temperature sterilized cow's milk containing 3% butterfat§ for 30 seconds. The expectorate was collected into a small cup and 3 ml immediately transferred to a disposable polystyrene test tube containing 0.12 ml of a 0.1% aqueous solution of methylene blue. The test tube was capped, mixed briefly, and allowed to stand over a mirror at room temperature. The time required for a color change from blue to white within a 6 mm diameter circle on the bottom of the test tube was recorded (Fig. 1). Pearson correlations between clinical parameters (PI and GI) and Oratest (i.e., the logarithm [base 10] of time required for the color change) were calculated. Spearman rank correlation analysis was also performed. Despite the ordinal nature of the clinical scores, both methods of analysis yielded essentially the same results.

RESULTS

A comparison of Oratest and PI indicated that the two parameters are related (i.e., in general, the higher the Oratest scores, the lower the PI). Results are summarized in Table 1 and Figure 2. When all 49 volunteers were grouped together, linear regression analysis of the logarithm of time required for color change, as a function of PI, yielded a correlation coefficient of −0.58 ($P = 0.001$). When the 19 volunteers who ate or drank within 90 minutes prior to the test were excluded (Fig. 2), the correlation coefficient rose to −0.64 ($P = 0.001$); the correlation coefficient of those who ate or drank within 90 minutes prior to the test was only −0.48 ($P = 0.019$).

As summarized in Figure 3 and Table 2, significant correlations ($P = 0.001$) were also observed between the log-

§Tnuva Ltd., Rehovot, Israel.

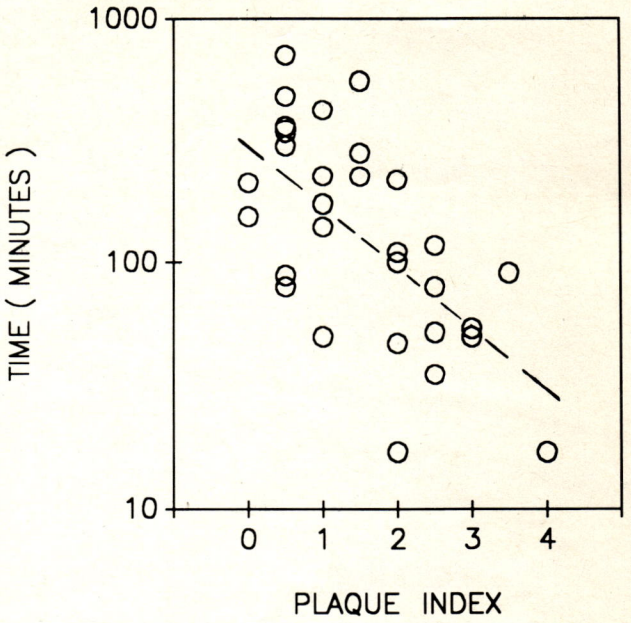

Figure 2: Correlation between Oratest results and Plaque Index. The time required for color change is plotted on a logarithmic scale as a function of the Plaque Index for the 30 volunteers who refrained from drinking or eating within 90 minutes prior to testing.

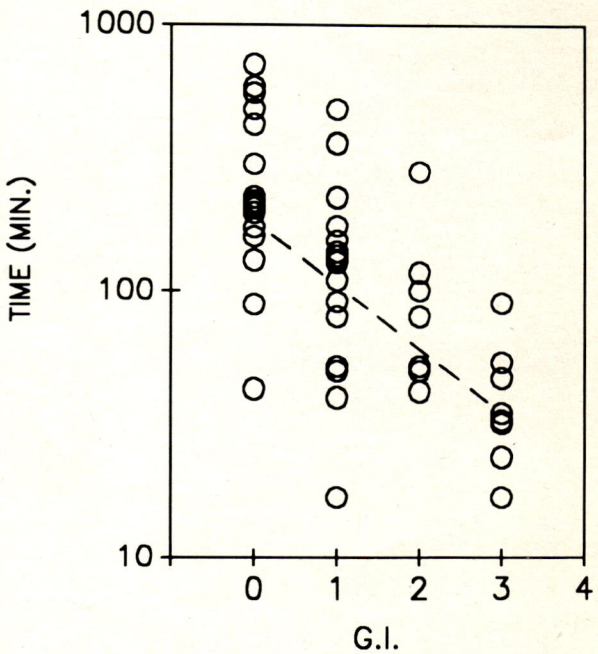

Figure 3: Correlation between Oratest results and Gingival Index. The time required for the color change is plotted on a logarithmic scale as a function of the Gingival Index for all 49 volunteers.

arithm of Oratest scores and GI values. These correlations were equivalent, whether or not the 19 volunteers who ate or drank within 90 minutes prior to the test were included (r = −0.66, and −0.65 respectively). The correlation coefficient obtained with those who ate or drank within 90 minutes of the test was −0.71 ($P = 0.001$).

Overall, Oratest scores varied greatly, from 17 to 585

Table 2. Relationship Between Oratest Result* and Gingival Index

Population	Number	Correlation Coefficient	Significance
All volunteers	49	$r = -0.66$	$P = 0.001$
Did not eat or drink†	30	$r = -0.65$	$P = 0.001$
Did eat or drink†	19	$r = -0.71$	$P = 0.001$

*Logarithm of time (minutes) required for blue-to-white color change at bottom of test tube.
†90 minutes prior to test.

minutes, with a mean of 178 minutes. The correlation between the logarithm of Oratest scores and increasing age of volunteers ($r = -0.34$; $P = 0.009$) was weaker than with either clinical parameter.

DISCUSSION

The growing interest in the microbiological aspects of periodontal diseases has led to the development of a variety of diagnostic approaches. Investigations have suggested relationships between the presence or level of certain microorganisms and evidence of periodontal diseases;[10] others have proposed to detect microbial products, such as proteolytic enzymes, or DNA.[12] Unfortunately, many of these approaches require extensive work-up time and additional equipment, including incubators and microscopes for morphological counts;[10] often samples must be sent out for analysis. Simple, inexpensive techniques which do not demand sophisticated skills or consume costly "chair" time, are required in order to expedite the diagnosis and appropriate management of periodontal diseases.

In a previous study, the Oratest was shown to be a simple, rapid technique for estimating oral microbial levels.[7] In the present study, the potential of the Oratest as a clinical diagnostic tool was examined. Consequently, correlations were sought between Oratest scores and commonly used clinical parameters (PI and GI). In both instances, significant ($P = 0.001$) overall correlations were observed between Oratest data and both PI and GI. Clearly, the subjective nature of PI and GI measurements themselves may have contributed to the somewhat weaker correlations observed here, as compared to the extremely strong correlations observed between Oratest scores and microbial counts.[7] In order to further establish the clinical significance of this technique in detecting progression of periodontitis, longitudinal studies comparing Oratest scores with loss of gingival attachment are required. In addition, the finding that the correlations are slightly altered by drinking or eating prior to the test warrants further investigation.

The data of the present study suggest that volunteers with high plaque levels almost invariably yield relatively rapid color changes in the Oratest. Thus, as can be observed in the upper right quadrant of Figure 2, not one of the volunteers with PI scores above 2 yielded color changes of longer duration than 120 minutes. Thus, Oratest scores of 2 hours or more may largely rule out the possibility of high plaque scores. On the other hand, Oratest scores of less than 2 hours may not necessarily indicate high plaque scores. Such "false positives" (i.e., suggesting a high oral microbial level, despite a low PI) may result from heavy colonization of buccal or tongue surfaces. Such conditions may predispose to other oral ailments, such as halitosis.[13] Moreover, soft oral surfaces may harbor periodontal pathogens.[14] In a separate study it was shown that baseline Oratest scores are significantly lengthened following the use of antibacterial mouthrinse.[15] Thus, one may speculate that patients exhibiting rapid Oratest color change, in spite of proper dental hygiene, are those who would benefit most from additional mouthrinse regimens.

The Oratest is based on whole mouth rinsing with sterile milk. We have previously proposed that milk is a suitable liquid for dislodging oral microorganisms mildly, yet effectively, while providing an excellent medium for subsequent metabolism.[7] Although whole mouth tests such as the Oratest do not identify the source of oral microorganisms, their relative simplicity and non-invasive nature make them potentially suitable for clinical use. Moreover, since whole mouth treatment regimens are routinely employed, whole mouth diagnostic tests may provide more relevant data than site-specific sampling techniques. Other potential advantages of the Oratest are: 1) it can be rapidly performed by auxiliary personnel; 2) positive results are easily visualized by both practitioner and patient; and 3) no special instrumentation is required.

Recent reports have suggested that the Oratest can serve as a simple, convenient technique to estimate oral microbial levels,[7] and to monitor mouthrinse regimens.[15] The present study extends these previous correlations to include two commonly used clinical parameters, PI and GI. Studies are underway to test the ability of the Oratest to provide a baseline with which subsequent changes in clinical status and oral hygiene (e.g., following scaling and instruction) can be monitored in a chairside, or even home, environment.

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Inhibition of the cooperative adhesion of *Streptococcus sanguis* to hydroxylapatite

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1. SUMMARY

The adhesion of *Streptococcus sanguis* to hydroxylapatite is a process involving several adhesins and receptors. Binding isotherms and Scatchard plots of the adhesion suggest that cooperative interactions occur at low cell densities. It was found that sulfolane, a hydrophobic-bond diluent, was capable of inhibiting the cooperative adhesion of *S. sanguis* to saliva-coated hydroxylapatite beads. Sodium thiocyanate, a chaotropic agent, inhibited not only cooperative adhesion, but also the adhesion thought to result from non-cooperative interactions. It is suggested that strong chaotropic agents may not only inhibit adhesin-receptor complexes, but also may influence the secondary/tertiary structures of interacting species.

2. INTRODUCTION

In the past few years, it has become increasingly clear that *Streptococcus sanguis* has multiple surface adhesins. Although none of these adhesins have been purified and completely characterized, there is evidence to suggest that the bacterium has one or more surface hydrophobic ligands (hydrophobins), polyelectrolytes and lectin(s). It is unknown if all potential adhesins are expressed coordinately. Nesbitt et al. [1] showed that isolates of *S. sanguis* tended to adhere to a hydrophobic surface prompting the suggestion that hydrophobic stabilization was important in adhesion of the bacterium to saliva-coated substrata. Several reports have emerged showing that streptococcal hydrophobic surface characteristics are needed for implanting the bacteria in experimental animals or for high-frequency caries formation [2–5]. Efforts have also been made to identify the protein(s) responsible for surface hydrophobicity in *S. sanguis* [6–11].

The adhesion of *S. sanguis* to saliva-coated hydroxylapatite beads (SHAB) appears to involve cooperative as well as non-cooperative interactions [1,2,9,10]. In cooperative adhesion, the ratio of bound (B) to unbound (U) cells increases as more cells become adherent. This report describes efforts to selectively inhibit cooperative adhesion. The results show that sulfolane, a non-denaturing

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hydrophobic solvent, can inhibit the adhesion of *S. sanguis* to SHAB at very low cell densities, where cooperative effects are most pronounced.

3. MATERIALS AND METHODS

3.1. Bacteria and growth conditions

S. sanguis 10556 was obtained from the American Type Culture Collection, Rockville, MD. The bacteria were maintained on brain heart infusion slants (Difco Laboratories, Detroit, MI) saturated with calcium carbonate. Cells were radiolabeled by growth in the presence of 8 μCi [^{3}H]thymidine per ml in brain heart infusion broth. Organisms from overnight culture at 37° in 5% CO_2 were harvested by centrifugation, washed twice and suspended in a mM potassium phosphate buffer containing 50 mM potassium chloride (pH 7.3) (PK buffer). Cells were gently sonicated prior to suspension to an absorbance of 2.0 at 540 nm (1-cm pathlength) [1,6,12].

3.2. Reagents

Sulfolane [13] (thiophene, tetrahydro-1,1-dioxide) was purchased from Aldrich Chemical Company, Milwaukee, WI. Reagent grade salts (sodium thiocyanate and buffer components) were obtained from Curtin-Matheson Chemicals, Cincinnati, OH. Tritiated thymidine (71 Ci/mmol) was from ICN, Irvine, CA. Spheroidal hydroxylapatite beads [14] were products of BDH Chemicals Ltd., Poole, U.K.

3.3. Saliva and bead preparation

Whole saliva was collected from laboratory personnel and centrifuged to remove particulate matter and cells. The hydroxylapatite beads were coated with saliva as described [1,6,12].

3.4. Adhesion assay

Radiolabeled cells (1.0 ml) were mixed with 40 mg hydroxylapatite beads in 15 ml plastic scintillation vials. The mixtures were rotated continuously for 90 min in order to attain equilibrium [12,15] and the beads separated by gravity. A 100 μl volume of cell suspension was decanted into scintillation vials to determine the unbound cell radioactivity. Following three washings with 3 ml of PK buffer, the beads containing adherent cells were counted by liquid scintillation. When it was necessary to use various cell densities, dilutions from the original cell suspension were made in buffer. Sodium thiocyanate or sulfolane (in PK buffer) was added to the beads prior to introducing the cells into the vials.

4. RESULTS

Fig. 1 shows the adhesion of *S. sanguis* to SHAB in the presence and absence of sulfolane. The results are presented in the form of binding isotherms. In the control, the curve appears to be sigmoid-shaped, with very little adhesion at low cell densities. At very high densities, saturation of sites did not seem to occur although there was a change in slope, suggesting an approach to saturation. In contrast, 1.0 M sulfolane inhibited adhesion at most of the cell densities employed. Sulfolane was most effective as an inhibitor at low cell densities, and in addition, tended to destroy the S-shaped binding profile. When the results were plotted using Scatchard's method, it was clear that sulfolane inhibited adhesion in the region where the B/U ratio usually increases with B (Fig. 2) (see also refs. 12 and 15). This region of

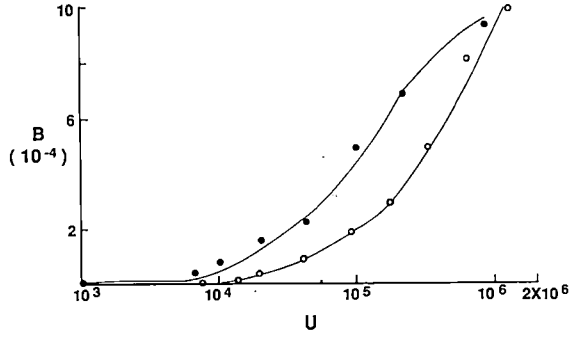

Fig. 1. Binding isotherms for the adhesion of *S. sanguis* to saliva-coated hydroxylapatite. Concentration of sulfolane was 1.0 M. The closed circle represents the control cell suspensions, whereas the open circle represents suspensions in the presence of sulfolane. B represents the bacteria bound to the hydroxylapatite. U signifies the proportion of cells which remained unbound. Units are in counts/min.

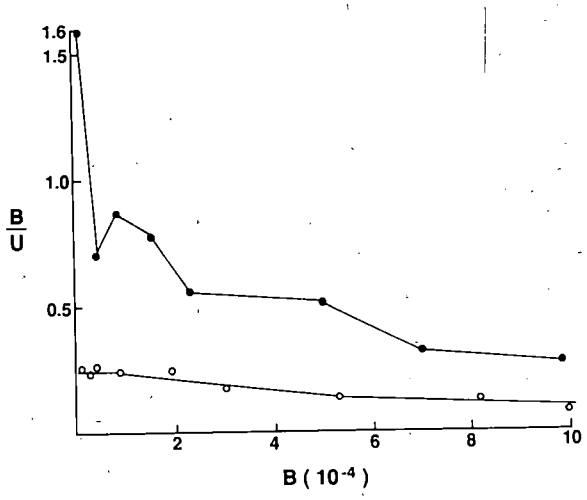

Fig. 2. Scatchard plot for adhesion of *S. sanguis* to saliva-coated hydroxylapatite. Data were derived from Fig. 1. Symbols are the same as in Fig. 1.

positive slopes is reflective of positive cooperativity.

When adhesion experiments were run in the presence of 1.0 M sodium thiocyanate, there was a general inhibition of binding at all cell densities (Fig. 3). Thiocyanate appears to be inhibiting cooperative as well as non-cooperative interactions. When 3.0 M urea, 3.0 M guanidine hydrochloride, 2.0 M sodium iodide or 10 mg/ml sodium dodecyl sulfate were employed as inhibitors the results

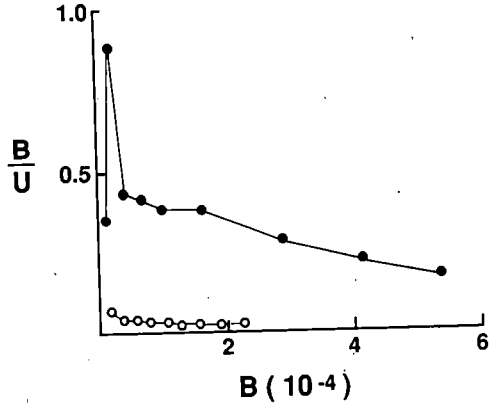

Fig. 3. Scatchard plot showing effect of thiocyanate on adhesion of *S. sanguis* to saliva-coated hydroxylapatite. Concentration of sodium thiocyanate was 1.0 M. Control suspensions indicated by closed circle; open circle represents cells suspended in thiocyanate.

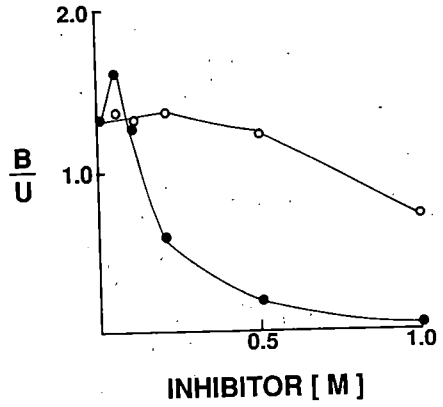

Fig. 4. Effects of sulfolane and thiocyanate anion on adhesion of *S. sanguis* to saliva-coated hydroxylapatite. A cell density of 0.05 absorbance units (a density expected to be involved in cooperative interactions) was chosen for all of the data points represented. Open circle represents sulfolane-treated cell suspensions, whereas the closed circle indicates the presence of thiocyanate.

were similar to those obtained with 1.0 M sodium thiocyanate (data not shown). Denaturants therefore tend to inhibit adhesion of *S. sanguis* to SHAB at all cell densities.

Interestingly, when a single low cell density of *S. sanguis* was employed, there was an enhanced adhesion at low concentrations of thiocyanate (Fig. 4). A 50 mM concentration of sodium thiocyanate gave a slightly increased B/U ratio whereas concentrations of sulfolane between 50 and 500 mM seemed to have little effect on adhesion.

5. DISCUSSION

Sulfolane has been reported to promote α-helix formation in proteins and to exhibit a tendency to solubilize hydrophobic proteins [13]. The protein-structure favoring qualities of sulfolane distinguish it from thiocyanate anion, a protein structure-destroying ion [16,17]. Vecchio et al. believe that sulfolane, at low to intermediate concentrations (5.0 M), can act as a non-denaturing solvent, capable of disrupting hydrophobic interactions, but with little effect on hydrogen bonds.

The inhibition of adhesion of the bacteria on the positive slope of the Scatchard plot suggests

that hydrophobic bonds may be involved in cooperativity. If this premise is correct, sulfolane may be interacting with newly-created hydrophobic sites. The initial attachment of the bacterium to artificial pellicle may involve ionic and dipole-dipole interactions, but once this adhesion has occurred, changes in the cell-pellicle junction may occur favoring hydrophobic interactions. These changes lead to cooperativity and are susceptible to hydrophobic bond diluents, such as sulfolane.

Results with thiocyanate and other protein structure breakers may be more easily understood. Thiocyanate could interfere with adhesin or receptor structure, solvent structure [16,17] or could solubilize salivary protein on the hydroxylapatite beads thereby removing potential cell receptors. It is likely that thiocyanate interfered with adhesion by a composite of its effects on adhesin(s), receptor(s) and solvent.

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Mechanism of Enhancement of Microbial Cell Hydrophobicity by Cationic Polymers

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Polycationic polymers have been noted for their effects in promoting cell adhesion to various surfaces, but previous studies have failed to describe a mechanism dealing with this type of adhesion. In the present study, three polycationic polymers (chitosan, poly-L-lysine, and lysozyme) were tested for their effects on microbial hydrophobicity, as determined by adhesion to hydrocarbon and polystyrene. Test strains (*Escherichia coli*, *Candida albicans*, and a nonhydrophobic mutant, MR-481, derived from *Acinetobacter calcoaceticus* RAG-1) were vortexed with hexadecane in the presence of the various polycations, and the extent of adhesion was measured turbidimetrically. Adhesion of all three test strains rose from near zero values to over 90% in the presence of low concentrations of chitosan (125 to 250 μg/ml). Adhesion occurred by adsorption of chitosan directly to the cell surface, since *E. coli* cells preincubated in the presence of the polymer were highly adherent, whereas hexadecane droplets pretreated with chitosan were subsequently unable to bind untreated cells. Inorganic cations (Na^+, Mg^{2+}) inhibited the chitosan-mediated adhesion of *E. coli* to hexadecane, presumably by interfering with the electrostatic interactions responsible for adsorption of the polymer to the bacterial surface. Chitosan similarly promoted *E. coli* adhesion to polystyrene at concentrations slightly higher than those which mediated adhesion to hexadecane. Poly-L-lysine also promoted microbial adhesion to hexadecane, although at concentrations somewhat higher than those observed for chitosan. In order to study the effect of the cationic protein lysozyme, adhesion was studied at 0°C (to prevent enzymatic activity), using *n*-octane as the test hydrocarbon. Adhesion of *E. coli* increased by 70% in the presence of 80 μg of lysozyme per ml. When the negatively charged carboxylate residues on the *E. coli* cell surface were substituted for positively charged ammonium groups, the resulting cells became highly hydrophobic, even in the absence of polycations. The observed "hydrophobicity" of the microbial cells in the presence of polycations is thus probably due to a loss of surface electronegativity. The data suggest that enhancement of hydrophobicity by polycationic polymers is a general phenomenon.

Hydrophobic interactions are considered to play a major role in a variety of microbiological phenomena, including adhesion (1, 8, 10–14, 17), phagocytosis (19), growth on insoluble substrates (16), and gliding (20). Whereas earlier studies suggested that each microbial strain possesses a fixed degree of surface hydrophobicity (19), more recent investigations have shown that physiological conditions (e.g., growth phase, medium, and presence of antibacterial agents) may often modulate hydrophobic surface properties (1, 2, 17). Cell surface components which promote (hydrophobins) or reduce (hydrophilins) hydrophobic properties may coexist on the cell surface (1, 14).

The hydrophobic interactions which mediate adhesion are often influenced by the composition of the aqueous medium. A wide range of components added to the aqueous phase can inhibit attachment to hydrophobic surfaces. These include amphipathic polymers (e.g., emulsan [12], surfactants [8, 10, 14, 16], and chaotropes [10]). However, few components have been found to promote attachment to hydrophobic surfaces. Ammonium sulfate, a salting-out agent, has been shown to enhance adhesion to hydrocarbons and polystyrene (13). Recently, E. Rosenberg and co-workers showed that the cationic antibiotic gramicidin S enabled *Bacillus brevis* to adhere to hexadecane (17). Certain cationic antibacterial agents (cetylpyridinium chloride and chlorhexidine) can also mediate adhesion to oil droplets (4a).

Cationic polymers, such as chitosan (2), lysozyme (18), and poly-L-lysine (7, 15), have been shown to promote microbial adhesion to various surfaces, although the mechanism has not been determined. The present report demonstrates that low concentrations of these polymers can confer microbial hydrophobicity, as determined by adhesion to hydrocarbons and polystyrene (12). This finding raises the possibility that microorganisms may acquire hydrophobic surface properties in the open environment by adsorbing cationic polymers.

MATERIALS AND METHODS

Strains and growth conditions. *Escherichia coli* CSH57 was obtained from E. Z. Ron, Tel-Aviv University; *Candida albicans* 792.1 was obtained from Y. Koltin, Tel-Aviv University. *Acinetobacter calcoaceticus* MR-481, a nonhydrophobic mutant of strain RAG-1, was isolated as described previously (16). Microorganisms were maintained on brain heart infusion agar plates (Difco Laboratories, Detroit, Mich.) at 4°C and transferred every month.

Polycations. Chitosan (practical grade; Sigma Chemical Co., St. Louis, Mo.) was prepared as previously described (6) by heating 0.5 g in 90 ml of 48% acetic acid until a solution

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TABLE 1. Effect of chitosan on microbial adhesion to hexadecane[a]

Strain	Initial turbidity (OD)[b]	Maximal adhesion (%)		Concn (μg/ml) of chitosan required for maximal adhesion
		Without chitosan	With chitosan	
E. coli CSH57	20	0	97	125
C. albicans 792.1	20	0	95	25
A. calcoaceticus MR-481	17	0	92	19

[a] Adhesion of washed microbial suspensions to hexadecane in the presence of various chitosan concentrations was carried out as described in Materials and Methods.

[b] Of washed cell suspensions before mixing in the presence of hexadecane.

TABLE 2. Effect of poly-L-lysine on microbial adhesion to hexadecane[a]

Strain	Initial turbidity (OD)	Maximal adhesion (%)		Concn (μg/ml) of poly-L-lysine required for maximal adhesion
		Without poly-L-lysine	With poly-L-lysine	
E. coli CSH57	22	0	59	625
C. albicans 792.1	18	0	96	200
A. calcoaceticus MR-481	13	0	98	125

[a] Conditions are described in the footnotes to Table 1.

was obtained and then dialyzing it extensively against distilled water. Poly-L-lysine hydrobromide (molecular weight, ca. 9,000) and chicken egg white lysozyme were obtained from Sigma.

Adhesion to hydrocarbon. Adhesion to hydrocarbon has been previously described (1, 11–14, 16, 17). In brief, microorganisms were grown from liquid inocula for 18 h with shaking at 30°C. Cells were washed twice and suspended in distilled water or distilled water containing 34 mM NaCl unless otherwise indicated. Turbidity of cell suspensions was measured at 400 nm in a Uvikon (Kontron, Zurich, Switzerland) 710 spectrophotometer. To semimicro, 1-cm^2, 4-ml disposable polystyrene cuvettes (Rudolf Brand, Wertheim, Federal Republic of Germany) containing 1.2 ml of cell suspension was added 0.4 ml of distilled water containing various polycation concentrations and other salts as indicated. *n*-Hexadecane or *n*-octane (0.2 ml) was added, and the cuvettes were vortexed for 60 s (sufficient for maximal adhesion) on a Thermolyne Maxi Mix flat-top mixer (Sybron, Dubuque, Iowa). Following phase separation, the turbidity of the lower aqueous phase (A_t) was measured directly in the cuvettes at 400 nm. For turbidity readings of over 1.0, a correction curve relating the observed reading to those of appropriately diluted suspensions was employed. Adhesion of cells to hexadecane was verified by microscopic observation. Results are expressed as the percent decrease in turbidity of the lower aqueous phase (A_t) compared with that of the original suspension (A_0), i.e., $100 - [100(A_t/A_0)]$.

Adhesion to polystyrene. Adhesion to polystyrene microdilution wells has been described elsewhere (13). Bacteria (*E. coli* CSH57) were washed and suspended in 34 mM NaCl to an optical density (OD) of 22. Different concentrations of chitosan were added to the bacterial suspensions, and 200-μl samples of the mixtures were added in quadruplicate to untreated flat-bottom polystyrene microdilution plates (Sterilin, Feltham, Middlesex, England) and incubated for 24 h at room temperature. Following incubation, the plates were rinsed thoroughly in running water. Adhering bacteria were then stained with gentian violet for 2 h at room temperature. To remove excess dye, plates were rinsed again in running water and allowed to dry. Adhesion was measured as the A_{610}, using a Dynatech Microplate Reader (Dynatech MR-600; Synatech Laboratories, Inc., Alexandria, Va.). As a control, 34 mM sodium chloride was substituted for bacterial cell suspension.

Chitosan pretreatment. In order to study the effect of chitosan on the cells and on the oil phase separately, *E. coli* CSH57 cells were washed and suspended to an OD of 20 in 34 mM NaCl. Following 60 s of agitation in the presence of chitosan (125 μg/ml, final concentration), the cells were washed twice and resuspended in 34 mM NaCl. Hexadecane (1.0 ml) was pretreated by vortexing (60 s) in the presence of 0.2 ml of aqueous solution containing 5 mg of chitosan per ml. The hexadecane droplets were then washed four times in distilled water. Adhesion to hexadecane was carried out as described above, employing pretreated versus untreated microbial cells and pretreated versus untreated hexadecane. As a control, adhesion of untreated cells to untreated hexadecane was brought about in the presence of 125 μg of chitosan per ml.

Chemical modification of cell surface. *E. coli* CSH57 was washed and suspended in 34 mM NaCl to an OD of approximately 20. The pH of the suspension was adjusted to 4.75 with dilute HCl. To 7 ml of bacterial suspension were added 15 ml of a 1 M aqueous solution of ethylenediamine (pH 4.75) and 9 ml of a 0.3 M aqueous solution of dicyclohexylcarbodiimide (pH 4.75) (3). Following 30 min of incubation at room temperature (pH was kept constant throughout), cells were washed three times and suspended in 34 mM sodium chloride to an initial OD of 17.5. Adhesion to hexadecane at various initial turbidities was carried out as described above.

RESULTS

The effects of chitosan and poly-L-lysine on microbial adhesion to hexadecane are summarized in Tables 1 and 2. Figure 1 compares the effect of the two polymers on adhesion of *E. coli* CSH57. In all cases, low chitosan concentrations enhanced adhesion to the hydrocarbon from 0 to over 90%; poly-L-lysine increased adhesion levels to 59 to 98%,

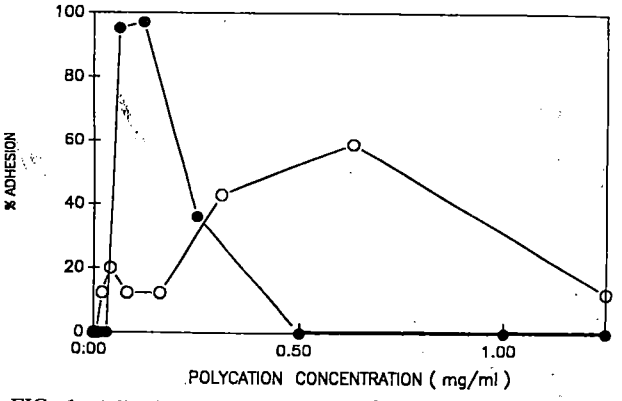

FIG. 1. Adhesion of *E. coli* (initial OD of 20) to hexadecane in the presence of chitosan (●) or poly-L-lysine (○). Adhesion of *E. coli* to hexadecane occurred in the presence of different polycation concentrations as described in Materials and Methods. Adhesion is expressed as the percent decrease in turbidity of the aqueous phase compared with the turbidity prior to mixing.

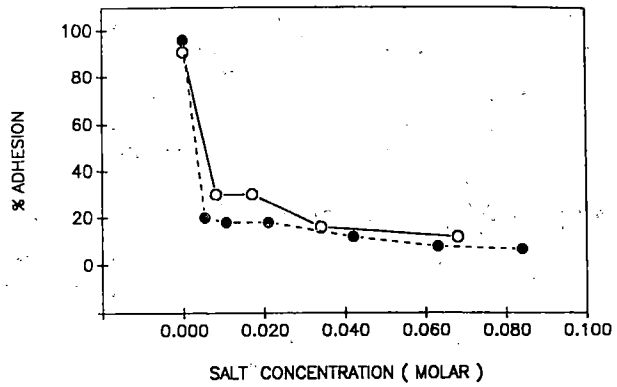

FIG. 2. Effect of salts on chitosan-mediated adhesion of *E. coli* to hexadecane. To 1.2 ml of cell suspension in distilled water at an OD of 20 was added 0.4 ml of distilled water containing chitosan to give a final concentration of 125 μg/ml and containing NaCl (○) or $MgCl_2$ (●) at various final concentrations as indicated.

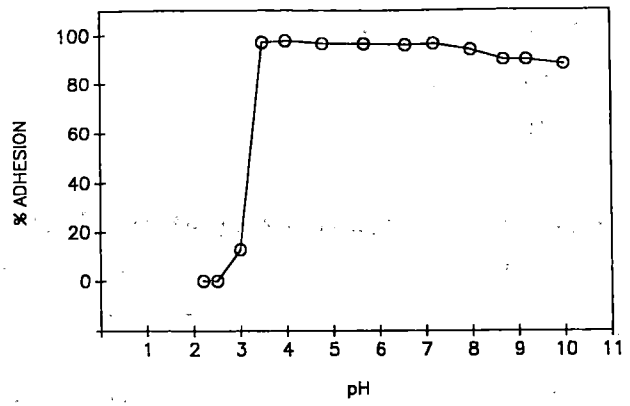

FIG. 3. Dependence of hydrogen ion on chitosan-mediated adhesion of *E. coli* to hexadecane. To 1.2 ml of washed cell suspension in 34 mM NaCl at an OD of 23 was added 0.4 ml distilled water containing HCl or NaOH to achieve the desired pH and chitosan to yield a final concentration of 125 μg/ml.

depending on the microbial strain. However, poly-L-lysine levels required for maximal adhesion (Table 2) were much higher than those observed for chitosan. For example, whereas 25 μg of chitosan per ml sufficed to increase the adhesion of *C. albicans* from 0 to 95% (Table 1), an eight-fold-higher concentration of poly-L-lysine was required to attain a similar adhesion level (Table 2). Moreover, whereas chitosan-mediated adhesion occurred over a very narrow range of polymer concentrations, the range of poly-L-lysine concentrations which promoted adhesion was much broader. Compared with poly-L-lysine, the corresponding monomer, L-lysine, was a poor inducer of hydrophobicity. Adhesion of *E. coli* cells was not enhanced by more than 15% over a wide range of L-lysine concentration (data not shown).

The strength of adhesion was assessed by measuring desorption of cells following centrifugation. Fewer than 10% of the *E. coli* cells bound to hexadecane in the presence of chitosan could be desorbed following 10 min of centrifugation at 1,100 × *g*.

The effects of salts on chitosan-mediated adhesion of *E. coli* to hexadecane is shown in Fig. 2. Both NaCl and $MgCl_2$ were highly inhibitory. For example, adhesion decreased by 70% in the presence of 140 mM NaCl and by 89% in the presence of 80 mM $MgCl_2$. The effect of pH on chitosan-mediated adhesion of *E. coli* to hexadecane is shown in Fig. 3. Values below pH 3.5 inhibited adhesion by over 80%.

In order to determine whether the adhesion-promoting effect occurred by partitioning of the polycation at the cell surface or at the hexadecane-water interface, *E. coli* cells and hexadecane droplets were independently pretreated with chitosan. Chitosan-pretreated cells adhered to hexadecane whether the hexadecane droplets were pretreated or not (89 and 91%, respectively). Similarly, adhesion in the control cuvette, in which untreated cells and hexadecane were mixed in the presence of chitosan, was high (98%). However, chitosan-pretreated hexadecane droplets were completely unable to bind untreated cells (0% adhesion). Thus, chitosan appears to promote adhesion by adsorbing directly to the microbial cell surface.

The effect of lysozyme on *E. coli* adhesion is shown in Fig. 4. In order to avoid enzymatic activity, adhesion experiments were performed at 0°C, using *n*-octane as the test hydrocarbon (hexadecane solidifies at approximately 16°C). Low concentrations of lysozyme (80 to 800 μg/ml) effectively promoted the adhesion of *E. coli* cells to octane. Lysozyme was also highly effective in promoting the adhesion of cells to hexadecane at room temperature (data not shown).

The adhesion of *E. coli* CSH57 to polystyrene as a function of chitosan concentration is shown in Fig. 5. Maximal adhesion was observed at 500 μg/ml. As with adhesion to hexadecane, high polycation concentrations resulted in a decrease in adhesion.

The foregoing data suggest that the microorganisms may be rendered hydrophobic by the complexing of electronegative sites by the polycations. One way to test this premise is to reduce the cell surface electronegativity independently of polycations. *E. coli* CSH57 was mixed with solutions of dicyclohexylcarbodiimide and ethylenediamine. Carbodiimide-activated carboxylate groups were substituted with positively charged ammonium groups from the ethylenediamine. When the derivatized cells were assayed directly for adhesion to hexadecane, it was found that there was now a high degree of binding to the hydrocarbon (Table 3). Adhesion was high over a wide range of initial cell densities. Controls performed in the presence of carbodiimide and ethanolamine

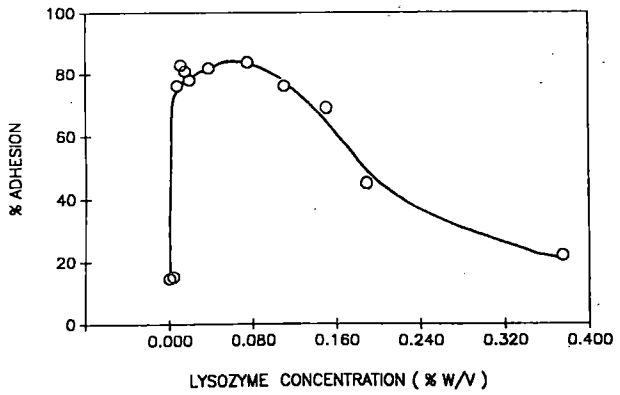

FIG. 4. Effect of lysozyme on adhesion of *E. coli* to *n*-octane. Adhesion of *E. coli* (at an initial OD of 22) to *n*-octane was carried out in the presence of different lysozyme concentrations as explained in Materials and Methods. Adhesion is expressed as the percent decrease in turbidity of the aqueous phase compared with the turbidity prior to mixing.

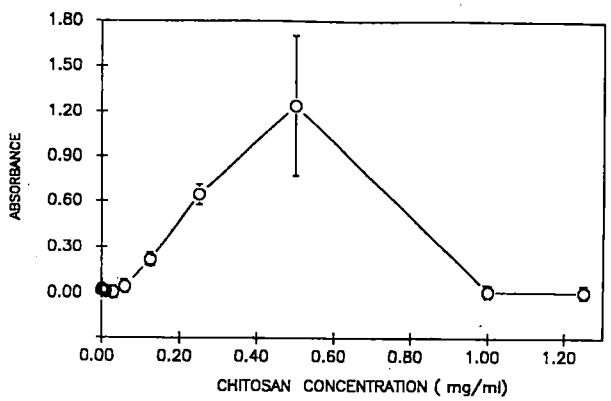

FIG. 5. Chitosan modifies adhesion of *E. coli* to polystyrene. Adhesion of *E. coli* to polystyrene microdilution wells was carried out as described in Materials and Methods. Results are plotted as the average absorbance (± standard deviation) as a function of chitosan concentration.

resulted in cells with intermediate adhesion properties (data not shown). Ethanolamine would give rise to the introduction of a neutral hydroxyl group instead of the positive ammonium residue.

DISCUSSION

The present study demonstrates that several polycationic agents (chitosan, poly-L-lysine, and lysozyme) confer hydrophobic properties on various microbial cells. Strains with no observable hydrophobic surface properties adhered avidly to hydrocarbon droplets in the presence of low polycation concentrations. At optimal concentrations, over 90% of the cells were removed from the bulk aqueous phase to the oil-water interface, as opposed to 0% in the absence of added polycation. Polycations, including those employed in the present study, have been shown to adsorb to solid surfaces and thus modulate the adhesion of microbial and mammalian cells. To our knowledge, this is the first report demonstrating their ability to mediate microbial adhesion at the oil-water interface.

The results suggest that polycations mediate microbial adhesion to hydrocarbon by binding to the cell surface via electrostatic interactions. This process is inhibited by salts and in some cases by protons. The adsorbed polymer may increase cell surface hydrophobicity by reducing the polar effects of negatively charged surface components. Concomitantly, outward orientation of hydrophobic residues present on the polymers may also increase cell-surface hydrophobicity. In the case of poly-L-lysine, the alkyl region of the side chain may contribute to hydrophobicity. Lysozyme is an amphipathic protein and has been shown to bind to hexadecane (15). In the case of chitosan, *N*-acetyl groups uncleaved by acidic hydrolysis may contribute to its hydrophobic properties.

TABLE 3. Adhesion of *E. coli* CSH57 to hexadecane following substitution of ammonium groups onto surface carboxylate residues[a]

Initial OD	% Adhesion
17.5	97
10	97
3.7	92
1.5	83
0.73	51

[a] *E. coli* was treated with dicyclohexyl carbodiimide and ethylenediamine as described in Materials and Methods. Following treatment, cells were washed three times and suspended in 34 mM NaCl to various initial turbidities, after which adhesion to hexadecane was determined. No adhesion to hexadecane was observed with untreated cells.

As we have demonstrated here, substitution of ammonium groups for carboxylate residues also promotes hydrophobicity in *E. coli*. Klotz and co-workers have reported increased hydrophobicity of *C. albicans* following blocking of carboxylate groups (9). Ferris and Beveridge have demonstrated that exposure of cation-depleted *E. coli* cells to magnesium ions resulted in increased hydrophobicity (4). E. Rosenberg and co-workers have previously shown that the cationic antibiotic gramicidin S confers adhesion to hexadecane on *B. brevis* cells (11). Goldberg et al. (4a) recently reported that cationic surfactants promote microbial hydrophobicity, as measured by adhesion to hexadecane and polystyrene. Similarly, foam flotation of microorganisms in the presence of cationic surfactants may be related to increased cell surface hydrophobicity (7, 8).

Together with the results of the present study, these data suggest a general microbial phenomenon by which a variety of nonadherent bacteria and yeasts can be rendered hydrophobic by a wide range of cationic agents. Moreover, the results presented here raise the possibility that a similar phenomenon occurs in vivo. The finding that lysozyme, a naturally occurring cationic polypeptide, mediates adhesion to hydrocarbons suggests that microorganisms may increase their cell surface hydrophobicity by adsorbing organic cations from the environment. Experiments to test this premise are under way.

In most instances studied here, higher concentrations of polycations brought about a decrease in adhesion to hydrocarbons and polystyrene. Similar observations have been noted for the effect of cationic surfactants on microbial adhesion to hexadecane (4a), foam flotation of *E. coli* (5), and flocculation of yeast cells (7). This phenomenon may be due to inhibition by unadsorbed polycations of cell-cell interaction. Cell-cell bridging may be of importance in stabilizing adhesion to hydrophobic substrata (M. Rosenberg, I. A. Buivids, and R. P. Ellen, manuscript submitted for publication). Indeed, polycation concentrations which enhanced adhesion in the present study frequently caused some microbial aggregation when added prior to the assay itself, in a manner similar to that observed for cationic surfactants (4a). Furthermore, the increase in percent adhesion of the chemically treated cells with increasing initial cell density (Table 3) suggests cooperative interactions among the adhering bacteria.

Microbial adhesion to hydrocarbons has frequently been used as a model for studying microbial cell surface hydrophobicity. A wide variety of bacteria and fungal cells have been shown to adhere avidly to hydrocarbons (14), yet many microorganisms of considerable scientific interest, such as *E. coli* and vegetative *Bacillus* cells, show little or no hydrophobic surface properties when tested by hydrocarbon adhesion and other hydrophobicity tests (13). The data suggest that hydrophobic properties can be conferred on such microorganisms by appropriate concentrations of cationic moieties, including nontoxic polymers such as chitosan. The potential effect of this acquired hydrophobicity on microbial adhesion and related phenomena warrants further study.

ACKNOWLEDGMENTS

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Effect of Cetylpyridinium Chloride on Microbial Adhesion to Hexadecane and Polystyrene

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Microbial adhesion at the oil-water interface is a subject of both basic interest (e.g., as a technique for the measurement of hydrophobicity) and applied interest (e.g., for use in two-phase oil-water mouthwashes for the desorption of oral microorganisms). In general, surfactants inhibit microbial adhesion to oils and other hydrophobic surfaces. In the present study, we demonstrated that the cationic surfactant cetylpyridinium chloride (CPC) significantly enhanced microbial adhesion to hexadecane and various oils, as well as to the solid hydrophobic surface polystyrene. CPC increased adhesion to hexadecane of *Escherichia coli*, *Candida albicans* and *Acinetobacter calcoaceticus* MR-481 and of expectorated oral bacteria from near 0% to over 90%. The CPC concentration required for optimal enhancement of adhesion was a function of the initial cell density. This phenomenon was inhibited by high salt concentrations and, in the case of *E. coli*, by a low pH. CPC-pretreated cells were able to bind to hexadecane, but CPC-pretreated hexadecane was unable to bind untreated cells. Another cationic, surface-active antimicrobial agent, chlorhexidine gluconate, was similarly able to promote microbial adhesion to hexadecane. The results suggest that (i) CPC enhances microbial adhesion to hexadecane by binding via electrostatic interactions at the cell surface, thus diminishing surface charge and increasing cell surface hydrophobicity, and (ii) this phenomenon may have applications in oral formulations and in the use of hydrocarbon droplets as a support for cell immobilization.

Over 60 years ago, Mudd and Mudd demonstrated that microorganisms exhibit considerable diversity in their affinity for the oil-water interface (5, 6). In recent years, adhesion to liquid hydrocarbons has become a commonly used technique for estimating hydrophobic surface properties of bacteria (10, 12). Applied aspects of microbial partitioning at oil-water interfaces include oil degradation (13), flotation of larvicidal spores (11), and desorption of bound bacteria from the oral cavity (14).

Surface-active agents generally act to inhibit microbial adhesion to hydrophobic surfaces. Examples include inhibition of bacterial adhesion to hydrocarbons by emulsan (8) and isopropanol (12) and inhibition of adhesion to plastics by chlorhexidine gluconate (CHX) (4, 15), Tween 20 (3), and Brij 56 (1).

In the present investigation, cetylpyridinium chloride (CPC), a cationic surfactant, significantly enhanced microbial adhesion to hexadecane. The data suggest that CPC can be used to promote microbial adhesion at the oil-water interface.

MATERIALS AND METHODS

Microbial strains and growth conditions. *Escherichia coli* CSH 57 was obtained from E. Z. Ron, Tel Aviv University; *Candida albicans* 792.1 was obtained from Y. Koltin, Tel Aviv University. *Acinetobacter calcoaceticus* MR-481, a nonhydrophobic mutant of strain RAG-1, was isolated as described previously (13). Microorganisms were maintained on brain heart infusion agar plates (Difco Laboratories, Detroit, Mich.) at 4°C and transferred every 2 months. For adhesion studies, microorganisms from several colonies were inoculated into test tubes containing 3 ml of brain heart infusion broth (Difco); following overnight growth with shaking at 30°C, cells were inoculated 1:200 into 250-ml Erlenmeyer flasks containing 50 ml of liquid medium of the same composition and grown with vigorous shaking for 18 h at the same temperature. Cells were washed twice and suspended in 0.2% NaCl or in distilled water for salt inhibition experiments (see Fig. 4). Expectorated oral bacteria were obtained from four adult volunteers who rinsed for 30 s with distilled water and then expectorated. The turbidity of cell suspensions was measured at 400 nm in a Uvikon 710 spectrophotometer (Kontron, Zürich, Switzerland).

Adhesion experiments. To 1-cm^2, 4-ml disposable polystyrene semimicrocuvettes (Rudolf Brand, Wertheim, Federal Republic of Germany) containing 1.2 ml of cell suspension was added 0.4 ml of distilled water containing various CPC concentrations and other salts as indicated. *n*-Hexadecane (0.2 ml) was added, and the cuvettes were vortexed for 60 s on a Thermolyne Maxi Mix flat-top mixer (Sybron, Dubuque, Iowa). In preliminary experiments (not shown), this duration of mixing was sufficient to attain maximal adhesion levels. Following phase separation, the turbidity of the lower aqueous phase was measured directly in the cuvettes at 400 nm. Turbidity readings of over 1 optical density (OD) unit were corrected for nonlinearity by interpolation by using a correction curve relating observed readings with actual values derived from appropriate dilutions falling within the linear range. Adhesion of cells to the hexadecane droplets was verified by microscopic observation (10). Results are expressed as the percent decrease in the turbidity of the lower aqueous phase (A_f) as compared with the turbidity of the original suspension (A_0), i.e., $100 - [100(A_f/A_0)]$.

Adhesion to polystyrene. Adhesion to polystyrene was tested essentially as previously described (9, 11). Bacteria (*E. coli* CSH 57) were washed and suspended in 0.2% NaCl

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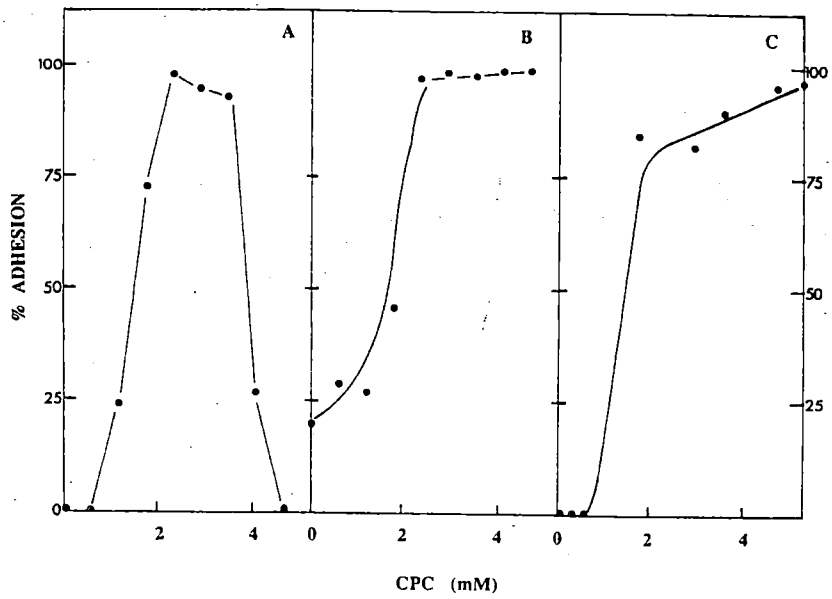

FIG. 1. Effect of CPC on microbial adhesion to hexadecane. Adhesion of *E. coli* (A), *C. albicans* (B), and *A. calcoaceticus* MR-481 (C) to hexadecane was tested in the presence of different CPC concentrations as described in Materials and Methods. Cells were washed twice and suspended in 0.2% NaCl to yield ODs of 24 (*E. coli*), 20 (*C. albicans*), and 11 (*A. calcoaceticus* MR-481). To 1.2 ml of cell suspension was added 0.4 ml of distilled water containing various CPC concentrations. Hexadecane (0.2 ml) was added, and the cuvettes were vortexed for 60 s. Adhesion is expressed as the percent decrease in the turbidity of the aqueous phase as compared with the turbidity before mixing.

to an OD of 22. Different concentrations of CPC (0.28 to 4.48 mM) were added to the bacterial suspensions, and 200-μl aliquots of the mixtures were added in quadruplicate to flat-bottom polystyrene microdilution plates (not tissue culture treated; Sterilin, Feltham, Middlesex, England) and incubated for 24 h at room temperature. Following incubation, the plates were rinsed thoroughly in a strong stream of running tap water normal to the plate surface. Adhering bacteria were stained with gentian violet for 2 h at room temperature. Plates were rinsed again in running water to remove excess dye and allowed to dry. Adhesion was measured as the A_{610} with an MR-600 Microplate Reader (Dynatech Laboratories, Inc., Alexandria, Va.). As a control, 0.2% NaCl was substituted for the bacterial cell suspension.

CPC pretreatment. To study the effect of CPC on the cells and on the oil phase separately, we washed and suspended *E. coli* CSH 57 cells to an OD of 20 in 0.2% NaCl. Following 60 s of agitation in the presence of CPC (2.24 mM final concentration), the cells were washed twice and suspended in 0.2% NaCl. Hexadecane (0.2 ml) was pretreated by vortexing (60 s) in the presence of 0.064 ml of an aqueous solution containing 2% CPC; the hexadecane droplets were washed four times in distilled water by successively replacing the lower aqueous phase with distilled water, followed by brief mixing. Adhesion to hexadecane was tested as described above with pretreated versus untreated microbial cells and pretreated versus untreated hexadecane. As a control, the adhesion of untreated cells to untreated hexadecane was tested in the presence of 2.24 mM CPC.

Chemicals. *n*-Hexadecane and *n*-octane were obtained from Fluka AG, Buchs, Federal Republic of Germany (Chemische Fabrik CH-9470). Mineral oil, commercial soybean oil, CPC, and CHX were obtained from Agis Industries, Ltd. (Tel Aviv, Israel). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.).

RESULTS

The effect of CPC on the adhesion of concentrated suspensions of *E. coli*, *A. calcoaceticus* MR-481, and *C. albicans* to hexadecane is shown in Fig. 1. In all cases, adhesion rose from low values to over 85% in the presence of CPC. Higher concentrations of the surfactant resulted in a decrease in the adhesion of *E. coli* but not of the other test strains. The effect of CPC on the adhesion to hexadecane of expectorated oral bacteria directly obtained from the oral cavity following rinsing is shown in Fig. 2. CPC (0.28 mM) increased adhesion from 29 to 79%.

The effect of CPC on the adhesion of *E. coli* was compared

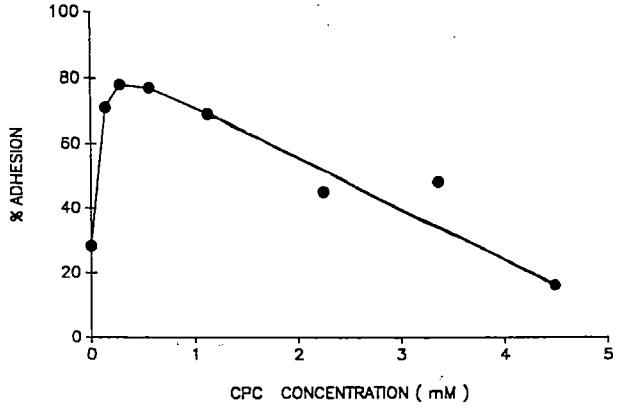

FIG. 2. Effect of CPC on the adhesion of expectorated oral bacteria to hexadecane. Expectorated oral bacteria were obtained from four volunteers. Distilled water (0.4 ml) containing various CPC concentrations was added to 1.2 ml of expectorated oral bacteria to a final OD of 1.9. Hexadecane (0.2 ml) was added, and adhesion was tested as described above.

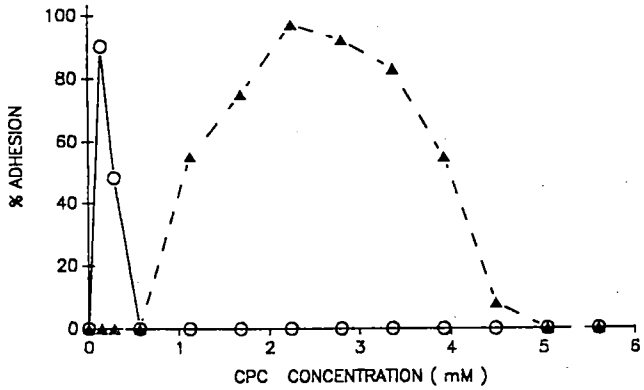

FIG. 3. Effect of CPC on the adhesion of *E. coli* at two different initial cell densities. The effect of CPC on the adhesion of *E. coli* to hexadecane was determined as described in the legend to Fig. 1 and in Materials and Methods. Cell suspensions were washed twice and suspended in 0.2% NaCl. To 1.2-ml aliquots of initial cell suspensions at ODs of 20 (▲) or 2.0 (○) was added 0.4 ml of distilled water containing various CPC concentrations, and adhesion to hexadecane was tested as described in Materials and Methods.

at two different cell densities (Fig. 3). At a lower initial cell density, much lower CPC concentrations (0.28 to 0.56 mM) were sufficient to bring about maximal adhesion. CPC (at a 2.24 mM final concentration) mediated adhesion of *E. coli* (initial cell density, OD of 20) not only to hexadecane (97%) but also to commercial soybean oil (97%), Sigma soybean oil (98%), mineral oil (96%), and octane (98%). No adhesion was observed when CPC was not present.

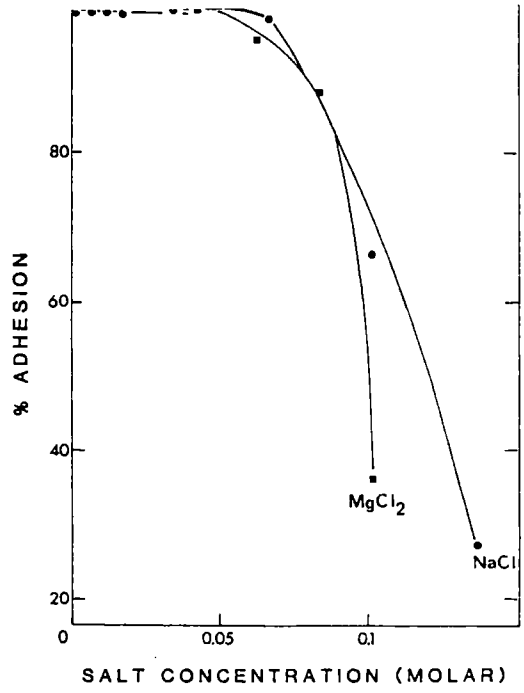

FIG. 4. Effect of salts on CPC-mediated adhesion of *C. albicans* to hexadecane. The adhesion of *C. albicans* to hexadecane was tested as described in Materials and Methods. *C. albicans* cells were washed twice and suspended in distilled water to an initial OD of 25. To 1.2 ml of aqueous cell suspension at an OD of 25 was added 0.4 ml of distilled water containing CPC to give a final CPC concentration of 4.48 mM and various final concentrations of NaCl or $MgCl_2$.

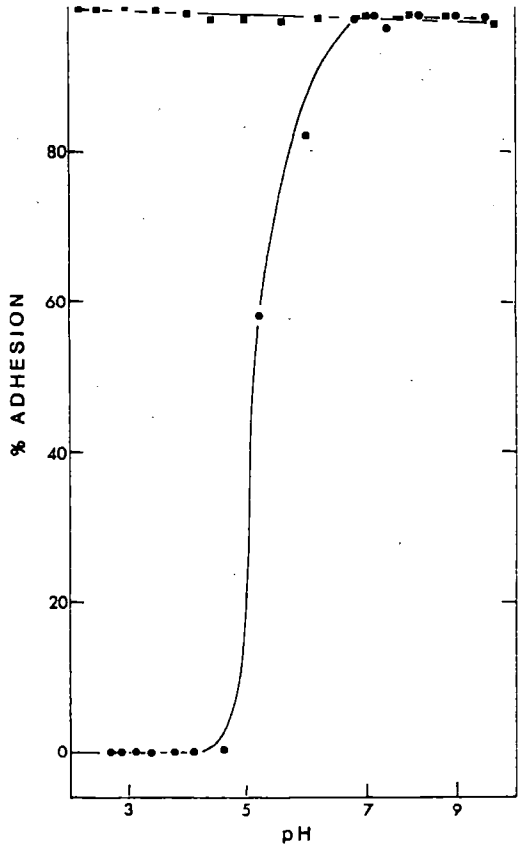

FIG. 5. Effect of pH on CPC-mediated adhesion of *C. albicans* and *E. coli* to hexadecane. To 1.2 ml of washed cell suspension in 0.2% NaCl was added 0.4 ml of distilled water containing CPC (2.24 mM final concentration), and HCl or NaOH was added to achieve the desired pH. The initial OD was 15 for both strains. Adhesion of *C. albicans* (■) and *E. coli* (●) to hexadecane was tested as described in Materials and Methods.

The effect of NaCl and $MgCl_2$ on the adhesion of *C. albicans* (Fig. 4) to hexadecane was studied in the presence of CPC. Increasing salt concentrations interfered with CPC-mediated adhesion. For example, adhesion dropped from 96% in the absence of NaCl to 27% in the presence of 0.14 M NaCl and from 99% in the absence of $MgCl_2$ to 36% in the presence of 0.11 M $MgCl_2$.

Figure 5 illustrates the effect of pH on CPC-mediated adhesion. Whereas a low pH completely interfered with the CPC-mediated adhesion of *E. coli*, the adhesion of *C. albicans* was not greatly affected over the pH range tested (2.2 to 9.2).

The effect of CPC on the adhesion of *E. coli* to polystyrene is shown in Fig. 6. The addition of 2.24 mM CPC increased adhesion 6.7-fold, as compared with the adhesion of the *E. coli* suspension containing no CPC. Higher concentrations of CPC resulted in a decrease in adhesion.

To determine whether the adhesion-promoting effect of CPC occurred by partitioning of the surfactant to the cell surface or to the hexadecane-water interface, we pretreated *E. coli* cells and hexadecane droplets separately with CPC (Fig. 7). CPC-pretreated cells adhered to hexadecane whether the hexadecane droplets were pretreated or not (98% in both cases). Similarly, adhesion in the control cuvette, in which untreated cells and hexadecane were mixed in the presence of CPC, was 98%. However, pre-

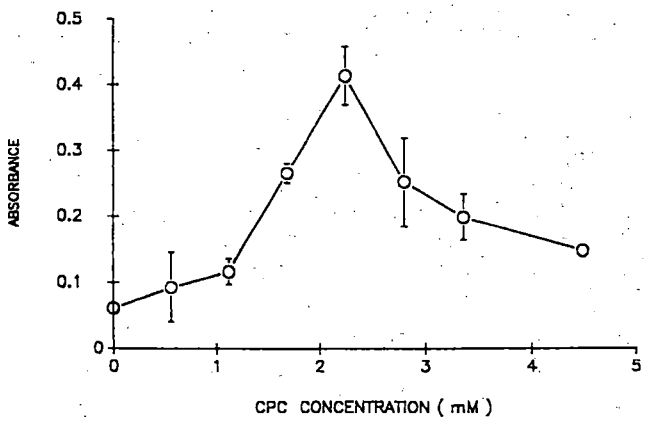

FIG. 6. Effect of CPC on the adhesion of *E. coli* to polystyrene. *E. coli* CSH 57 cells were washed and suspended in 0.25 NaCl to yield an OD at 400 nm of 22. Adhesion to polystyrene was tested as described in Materials and Methods.

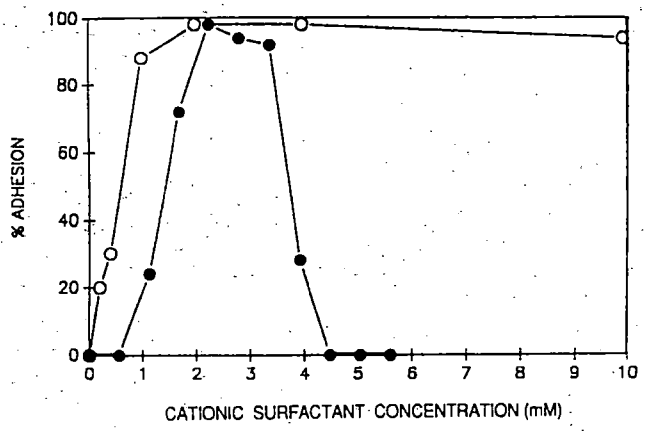

FIG. 8. Effect of CHX (○) and CPC (●) on the adhesion of *E. coli* to hexadecane. The effect of increasing concentrations of CHX and CPC on the adhesion of *E. coli* CSH 57 to hexadecane was determined as described in Materials and Methods and in the legend to Fig. 1.

treated hexadecane droplets were unable to bind significant quantities of untreated cells (3%).

The ability of another cationic surface-active antibacterial agent, CHX, to promote adhesion to hexadecane is shown in Fig. 8.

DISCUSSION

The results of this study show that CPC, a cationic surfactant commonly used as an antibacterial agent, greatly enhances microbial adhesion to hexadecane and to the hydrophobic test surface polystyrene. The three microbial strains used do not normally adhere at the hexadecane-water interface; indeed, *A. calcoaceticus* MR-481 is a nonhydrophobic mutant originally selected from adherent RAG-1 cells by its inability to adhere to *n*-octane (13). Adhesion of all three strains to hexadecane reached levels of over 90% at optimal CPC concentrations. CPC similarly enhanced the adhesion of expectorated oral bacteria to hexadecane and promoted the adhesion of *E. coli* cells to polystyrene. This phenomenon was not observed with nonionic or anionic surfactants.

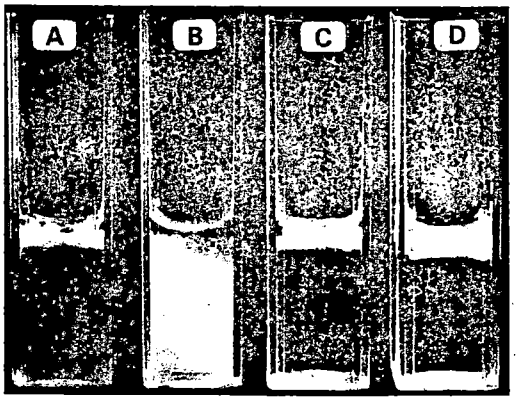

FIG. 7. Effect of CPC pretreatment of cells and hexadecane on the adhesion of *E. coli*. Pretreatment of *E. coli* cells with CPC and pretreatment of hexadecane with CPC were done as described in Materials and Methods. Following pretreatment, cuvettes were vortexed in the presence of treated or untreated hexadecane for 60 s. (A) Adhesion of CPC-pretreated *E. coli* cells to untreated hexadecane. (B) Lack of adhesion of untreated *E. coli* cells to CPC-pretreated hexadecane. (C) Adhesion of untreated *E. coli* cells to CPC-pretreated hexadecane. (D) Adhesion of untreated *E. coli* cells to untreated hexadecane in the presence of 2.24 mM CPC.

The data suggest that CPC acts to enhance adhesion by binding directly to the cell surface, as (i) CPC-pretreated cells adhered to hexadecane but CPC-pretreated hexadecane did not bind bacteria and (ii) the optimal CPC concentration for adhesion was a function of the initial cell density.

In most adhesion studies, increasing salt concentrations promote adhesion, presumably by decreasing electrostatic repulsion and by ion bridging. In this study, the addition of NaCl and $MgCl_2$ acted to prevent CPC-enhanced adhesion. Apparently, the surfactant acts by binding via its positive charge to negatively charged groups at the cell surface; this binding is inhibited by the presence of other cations (Na^+, Mg^{2+}), which compete for the negatively charged groups. In some instances (e.g., CPC-mediated adhesion of *E. coli* to hexadecane), a low pH may also interfere by reducing the number of free anionic surface moieties. The exposed hydrophobic moiety of the bound CPC molecules can then increase cell surface hydrophobicity, enabling partitioning of the cells at the oil-water interface; the reduction in the net negative surface charge of the cells may also contribute to the hydrophobic properties observed. A similar mode of action has been proposed for the increase in the hydrophobicity of mutant *Bacillus brevis* spores brought about by the addition of the antibiotic gramicidin (7). In addition, binding of CPC to the cell surface may promote cell-cell bridging, which may stabilize adhesion. Indeed, optimal CPC concentrations for enhanced adhesion brought about some aggregation when added prior to mixing with hexadecane. The drop in CPC-mediated adhesion to hexadecane and polystyrene at higher CPC concentrations may have been due to inhibition of the cell-cell interaction by free CPC molecules. Kamada and Murata similarly observed that, whereas at low concentrations cetyltrimethyl ammonium bromide flocculated yeast cells, at higher concentrations it acted as a deflocculant (2).

CPC-mediated enhancement of microbial adhesion to hexadecane appears to represent a general phenomenon: (i) certain other cationic antibacterial agents, e.g., CHX, are also capable of mediating adhesion to hexadecane; (ii) CPC enhances adhesion not only to hexadecane but also to a

variety of oils and to polystyrene; (iii) even adhesion of hydrophobic strains, such as *A. calcoaceticus* (RAG-1), to hexadecane can be increased by CPC (Y. Konis and M. Rosenberg, unpublished data).

Enhanced adhesion of microorganisms to oil-water interfaces has a number of potential applications (10, 11, 13). Previous investigations in this laboratory have led to the suggestion that two-phase, oil-water mouthwashes can efficiently desorb oral microorganisms by preferential adsorption to the oil droplets (14). The results presented here show that partitioning of oral bacteria, obtained directly from the oral cavity, at the oil-water interface is greatly enhanced by CPC. Indeed, the present investigation was initiated to test how CPC, a cationic surfactant commonly used in oral rinses, affects microbial adhesion to oil. The data presented suggest that CPC may enhance the ability of oil-water formulations to bind and remove microorganisms from the oral cavity. Further studies in this direction are under way.

ACKNOWLEDGMENTS

We thank Zinaida Zosim and Ronit Bar-Ness for reviewing the manuscript and for helpful discussions. We are grateful for the excellent technical help of Yardena Mazor, the photographic assistance of Lita Maman, and the editorial assistance of Rita Lazar.

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tion was primarily a shrinkage in the occlusal dimension, expansion in the frontal dimension, and shrinkage and expansion alike in the lateral dimension.

3. The mean positional changes generally fell below 1% in the occlusal dimension but were much larger in the frontal and lateral dimensions, ranging from 0.2% to 8.1% frontally and 0.2% to 9% laterally.

4. Significant differences between resin systems were observed in mean positional changes in all three dimensions.

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An innovative method of monitoring denture hygiene

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Tel Aviv University, The Maurice and Gabriela Goldschleger School of Dental Medicine, Tel Aviv, Israel

A simple and rapid test for measuring oral hygiene was recently developed. It is based on the rate of oxygen consumption of oral expectorates of milk. This investigation modified the test to study denture hygiene. The dentures of 20 patients were immersed in 10 mL of sterile milk. After a 2-minute agitation, 3 mL of milk was added to test tubes containing methylene blue. The time required for color change at the bottom of the test tube, which is indicative of the rate of oxygen consumption, was recorded. For comparison with visual plaque accumulation, the dentures were coated with disclosing solution and the extent of plaque was scored by three examiners. A correlation was found between the plaque index scores and results of the milk test ($r = -0.64$; $p < 0.005$). The data suggest the use of this test to monitor denture hygiene. (J PROSTHET DENT 1990;63:661-4.)

Soft tissue changes associated with unclean removable dentures are denture stomatitis, inflammatory papillary hyperplasia, and chronic candidiasis.[1-6] Since most of the causative microorganisms in denture stomatitis reside on the denture, disinfection of the denture is an essential preventative procedure.[7-9]

The extent of plaque on dentures can be demonstrated by dye binding (disclosing) techniques[10] and assessment of specific organisms obtained by plating oral samples on selective growth media.[9] Residual plaque accumulations after use of denture cleansers have been examined with a scanning electron microscope.[11, 12] Altman et al.[13] critically evaluated methods to determine the cleanliness of dentures and suggest a spectrofluorometric protein assay of plaque as a most useful guide to denture cleanser efficacy.

This study was carried out in the Alpha Omega Research Laboratories, supported by the Alpha Omega Foundation.

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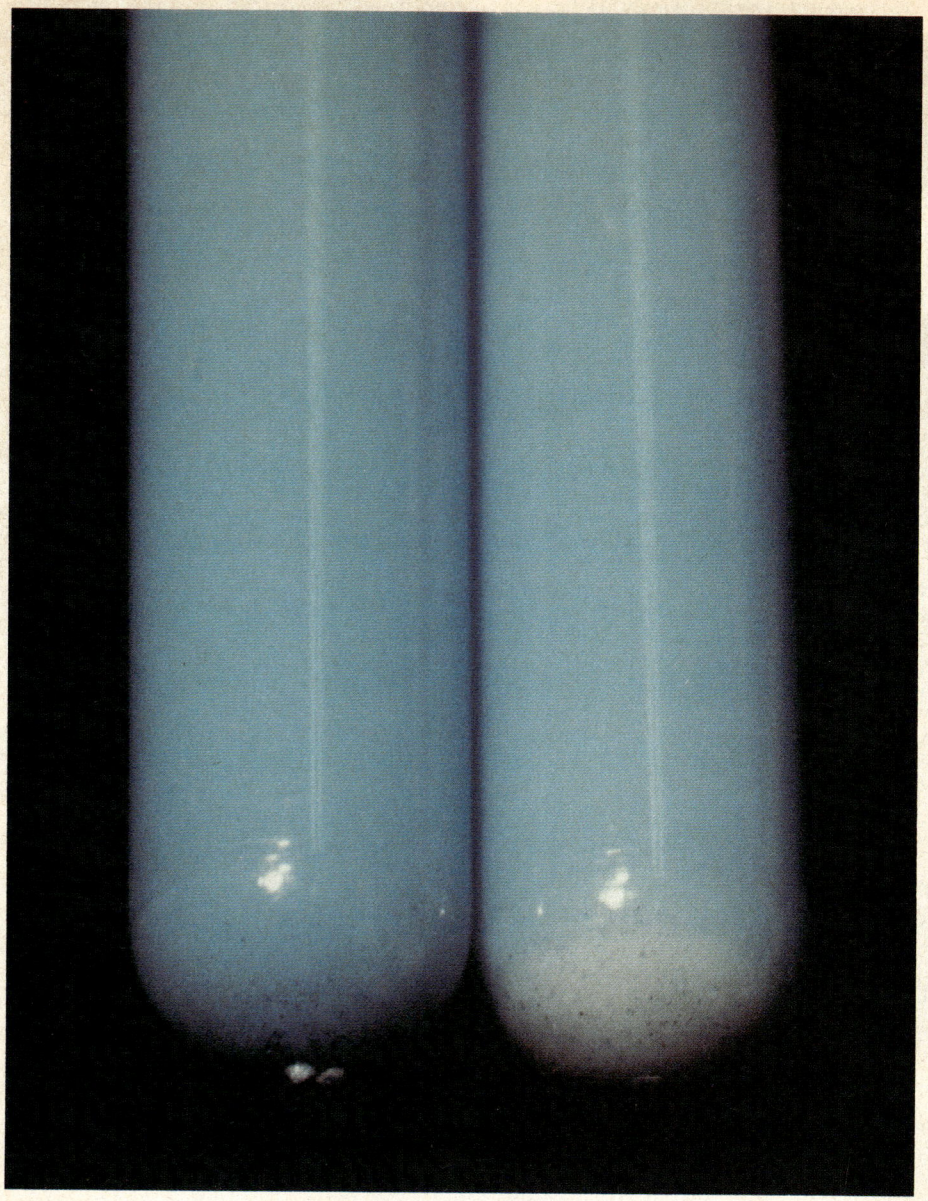

Fig. 1. Color change occurring at base of test tube.

Tarbet[10] demonstrated that a rigorous denture hygiene program reduced the level of denture plaque and improved irritated mucosa. Rosenberg et al.[14] recently developed a simple test for measuring oral hygiene. Basically, the patient rinses vigorously for 30 seconds with sterile milk, which is then expectorated. The level of microorganisms in the expectorate is then assessed by the rate of oxygen consumption, with methylene blue as a redox indicator.

The test was modified in this study to determine its usefulness in monitoring denture hygiene.

MATERIAL AND METHODS

Twenty patients 52 to 73 years of age were selected. They had been wearing either maxillary and mandibular complete dentures or maxillary complete denture and mandibular removable partial denture replacing 10 teeth for more than 1 year.

The dentures were placed in a plastic bag containing 10 mL of ultrahigh temperature (UHT) sterilized commercial cow's milk with 3% butterfat (Tnuva, Rehovot, Israel) and agitated for 2 minutes. Three mL of milk were removed and added to 0.12 mL of methylene blue in a sealed polystyrene test tube. The test tube was agitated for 5 seconds and allowed to stand at room temperature. The time required for a color change from blue to white over a 6 mm diameter at the bottom of the tube was recorded (Fig. 1).

Plaque was identified on the tissue surface of the dentures by applying a disclosing solution (Red Cote, J.O.

Table I. Mean plaque scores and time of color change in test solution

Patient	Mean total plaque score	Time for color changes (minutes)
1	14.0	180
2	15.7	120
3	14.3	150
4	17.3	7
5	29.0	30
6	16.7	60
7	13.7	90
8	8.7	720
9	12.3	420
10	10.3	300
11	19.0	45
12	13.0	90
13	19.0	45
14	16.0	60
15	10.0	360
16	20.0	45
17	14.3	25
18	14.0	30
19	9.6	120
20	20.0	60

Fig. 2. Disclosed dentures. Total plaque score of 26 was recorded.

Butler Co., Chicago, Ill.). The disclosed dentures were photographed, then scrubbed with a toothbrush, soap and water, washed, reimmersed in a milk solution, and retested. The time required for color change at the bottom of the test tube was again recorded. Color slides of the dentures were independently inspected by three examiners and plaque scores were recorded.

Plaque scoring was done in a manner similar to that described by Tarbet.[10] The tissue surface of the disclosed dentures was divided into approximately equal quadrants by mentally drawing a line anteroposteriorly through the midline and another line perpendicular to it at approximately the premolar region. Plaque scoring was:

0 = No plaque
1 = 25% or less of the quadrant covered by plaque
2 = 26% of the quadrant covered by plaque
3 = 51% to 75% of the quadrant covered by plaque
4 = 76% to 100% of the quadrant covered by plaque

The total of the quadrant scores gave the total plaque score of the denture (Fig. 2).

RESULTS

Table I shows the combined plaque scores for each set of dentures and the time recorded for the color change of the liquid. The graph (Fig. 3) compares the logarithm of the time recorded for color change with the plaque score; linear regression indicated a good correlation ($r = 0.64$; $p < 0.005$). In all instances, after the denture was cleaned no color change occurred for up to 8 hours, indicative of low microbial levels.

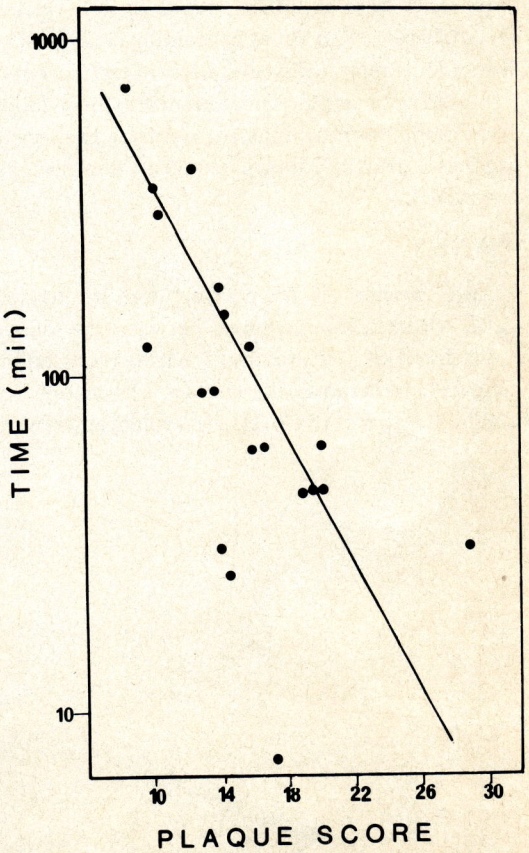

Fig. 3. Relationship between plaque scores and logarithm of time recorded for color change.

DISCUSSION

Many patients are not informed on how to care for their removable dentures. When instructions are given, cleaning is often done in a perfunctory and incomplete manner. Whereas the teeth and the polished surface of the dentures are usually brushed, the tissue surface is given scant attention. Older denture patients may have reduced manual dexterity for cleaning dentures and less than acute eyesight to detect the almost invisible layer of plaque on the acrylic resin. Examination of the disclosed dentures revealed that most plaque accumulated in the undercut regions of the labial flanges not easily accessible to the toothbrush.

All patients questioned in the study thought that their denture hygiene was adequate and expressed surprise when confronted with disclosed dentures. A high level of interest was shown in the milk test. The technique was quickly grasped by the patients who were informed that a change in color occurring in less than 4 hours (approximately equivalent to a plaque score of 10) indicated unclean dentures.

The suggestion that the patient could monitor their own denture hygiene at home was not greeted enthusiastically, but when given a choice of method, all patients preferred the milk test as it appeared less messy to use than disclosing solution.

The patients were supplied with denture hygiene kits consisting of plastic test tubes containing 0.12 mL methylene blue and line plastic pipettes. Most of the patients were being treated with replacement dentures and follow-up tests were made on the old dentures during their visits. In all instances a dramatic improvement in denture hygiene was observed.

SUMMARY

A simple method of monitoring denture hygiene is described. A correlation is shown between the amount of plaque disclosed on dentures and the time required for the test solution to undergo a color change. The change in color is a visual indication of the level of microbial activity within the liquid in which the dentures were immersed. Patients may be supplied with home denture hygiene kits to monitor denture cleanliness.

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MICROBIAL ADHESION TO HYDROCARBONS (MATH)

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Microbial adhesion at the oil:water interface has been studied since the pioneering work of Mudd & Mudd (1924) in various contexts. A decade ago, microbial adhesion to hydrocarbons (MATH) was proposed as a general test for cell-surface hydrophobicity. Since that time, various modifications, including a kinetic approach, have been adopted in different laboratories. The MATH test has helped elucidate surface structures which promote (hydrophobins) or reduce (hydrophilins) the hydrophobic properties of the outermost cell surfaces of various microorganisms. In two Gram negative bacteria, *Serratia marcescens* and *Acinetobacter calcoaceticus*, a wide array of components differing greatly in composition, appear to influence adhesion to hydrocarbon. Many microorganisms exhibit little or no affinity for the water:hydrocarbon interface. However, addition of appropriate concentrations of cationic molecules (cetylpyridinium chloride, chlorhexidine, chitosan, poly-L-lysine and lysozyme) can hydrophobize microorganisms and potentiate their adhesion. Several recent observations raise the possibility that microbial adhesion to hydrocarbons is, in certain cases, cell-concentration dependent. This may reflect the increased stabilization of hydrocarbon droplets by sufficient numbers of interacting cells.

KEY WORDS: Hydrophobicity, microorganisms, adhesion, hydrocarbon, cooperativity.

INTRODUCTION

Bacterial adhesion to hydrocarbons (BATH) was proposed in 1980 as a simple and convenient assay for measurement of cell surface hydrophobicity (Rosenberg *et al.*, 1980). Since that time, this technique and its modifications have been used extensively to study cell surface properties of a wide range of bacteria. More recently, the assay has also been employed to study fungal hydrophobicity; thus the acronym MATH (microbial adhesion to hydrocarbons) is currently employed (Rosenberg & Doyle, 1990). The purpose of the present overview is (i) to present a brief, critical review of the original technique and the more recent kinetic approach; (ii) to discuss some generalizations regarding cell surface components which promote or interfere with microbial adhesion to hydrocarbons; (iii) to review recent data suggesting that classes of cationic molecules can mediate adhesion of a wide range of microorganisms to the oil:water interface, and (iv) discuss the potential role of droplet stabilization in the observed phenomenon.

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THE ORIGINAL ASSAY AND THE KINETIC APPROACH

According to the basic MATH assay, turbid washed suspensions of microbial cells (1·2 ml) are vortexed in the presence of 0·01–0·2 ml of liquid test hydrocarbon (*n*-hexadecane, *n*-octane, *p*-xylene). During the mixing procedure, the liquid hydrocarbon is dispersed into droplets which can adsorb microbial cells. In the case of nonadherent cells, the droplets quickly coalesce, the turbidity in the lower aqueous phase remains unchanged, and the contents of the test tube appear to have been unaffected by the procedure. When adhesion takes place, the cell-coated droplets rise to form a stable "cream". Phase separation is usually complete within one or two minutes. The extent of adhesion to the hydrocarbon droplets can be simply ascertained by the decrease in cell-mediated turbidity in the lower aqueous phase. Originally, the lower aqueous phase was withdrawn using a Pasteur pipette and transferred to a semi-micro cuvette for measurement of optical density at 400 nm (Rosenberg *et al.*, 1980). However, the assay can be performed directly within a cuvette or tube that is compatible with a spectrophotometer, in which case the decrease in turbidity can be read directly, provided that care is taken to ensure that the upper phase does not interfere with the light beam and that the walls of the test tube or cuvette are free of interfering droplets.

The assay has become popular because it is so simple to perform, and the necessary instruments (vortex type mixer, test tubes, photometer) are close at hand in practically every microbiological laboratory. This, however, is related to the assay's main drawback. Since mixers, test tubes and methods of turbidimetric measurements vary from laboratory to laboratory, the results obtained in one laboratory cannot necessarily be compared to those obtained in another. Furthermore, individual laboratories often change one or more aspects of the assay to suit the question they are addressing. Over the past decade, several modifications in the basic assay have been adopted by many laboratories using the test. The original PUM buffer is often replaced by P buffer (*i.e.* 150 mM phosphate buffer without the urea and magnesium sulphate) (Rosenberg & Doyle, 1990). Originally, the test tubes were preincubated at 30°C prior to the assay. This step also has been abandoned largely, despite its potential importance in improving reproducibility. Clearly, performing the test with n-hexadecane, at room temperatures close to its solidification point (16°C) may have a large effect on droplet size, a crucial aspect of the assay. Whereas the original assay was based on results obtained with three test hydrocarbons, the assay is often currently employed using a single hydrocarbon (usually hexadecane). Recently, Vanhaecke and Pijck (1988) showed that hexadecane does not damage the intactness of the cells during the assay; similar results were obtained with octane. *p*-Xylene, on the other hand, resulted in cases of cell lysis and its use as test hydrocarbon should be avoided, whenever possible.

The original assay was performed with test tubes which had been acid-washed. Since surfactants interfere with the assay (Rosenberg *et al.*, 1980), care must be taken in using clean utensils. The authors have recently found that commercially-purchased *n*-hexadecane (including those stated to be >99% pure) may contain contaminants that interfere with the assay. However, n-hexadecane recently purchased from Sigma Ltd (St Louis) gave highly reproducible results. Hexadecane can be protected from undesirable oxidation by storage under nitrogen gas. In any case, a simple test can ascertain whether the assay is relatively free of surfactants: the buffer and test hydrocarbon are vortexed under the assay conditions. If the droplets do not coalesce rapidly, or if a stable emulsion is formed, an intefering contaminant is present.

The MATH assay has several inherent advantages, in addition to its basic simplicity. Since it is a rapid test, there is little chance for the microorganisms to undergo cell-

surface changes as a result of ongoing metabolism. Secondly, the adherent cells rise following the assay, so there is little chance of confusing settling or clumping of cells with adhesion (Rosenberg & Doyle, 1990). Another advantage is the possibility of direct observation of the cells adhering to the substratum in the phase microscope (or even the light microscope, following addition of a stain, such as gentian violet, which enhances contrast [Rosenberg, 1990]). Adhesion to hexadecane can also be observed by electron microscopy (Rosenberg *et al.*, 1991, submitted). A further advantage of the assay is that no washing steps are necessary to distinguish adherent and nonadherent cells. Washing can result in desorption or translocation of cells, particularly if air: stratum interfaces are generated during the washing procedure (H Busscher, personal communication). Finally, in contrast to many assays employing solid substrata in which only a small fraction of the cell suspension actually comes in contact with the surface, in the MATH assay the adhesion characteristics of the entire suspension are measured.

In the original assays, test tubes were vortexed for a standard duration (2 min), and percent adhesion was followed as a function of varying hydrocarbon volumes (Rosenberg *et al.*, 1980). An optimal quantitative assay would entail mixing until steady-state conditions are attained. However, the more one mixes, the smaller the droplets, and the greater the surface available for adhesion. This prompted several researchers to propose a kinetic approach (Lichtenberg *et al.*, 1985; Sharon *et al.*, 1986) in which adhesion to increasing hydrocarbon volumes is followed as a function of time. This modification yields removal rates for each hydrocarbon volume. Plotting of the removal rates as a function of the hydrocarbon:water volume ratio yields a straight line, the slope of which is termed the "removal coefficient". In most instances studied to date, back extrapolation of this line to a hydrocarbon:water volume of zero (*i.e.* no hydrocarbon) does not yield a zero adhesion rate, as one would expect, but rather a finite value. This phenomenon has yet to be adequately explained (Sharon *et al.*, 1986). Another potential problem is that, at least in the case of *Acinetobacter calcoaceticus* RAG-1, the kinetics of adhesion are dependent on the initial cell concentration (M Barki, unpublished).

MATH is one of several commonly employed tests for measuring the amphipathic nature of microbial cells. Other examples include contact-angle measurement (CAM), hydrophobic interaction chromatography (HIC), and the salt aggregation test (SAT). The degree of correlation between these assays is a matter of some controversy. In many, but not all instances, results obtained using MATH correlate with other putative hydrophobicity tests (Rosenberg & Doyle, 1990), particularly when closely-related strains are compared (van der Mei *et al.*, 1987).

CELL SURFACE COMPONENTS INVOLVED IN ADHESION TO HYDROCARBONS

Initially, it was thought that the hydrophobic surface properties of any given strain could be related to a single cell surface component. In certain instances, *e.g.* the A protein layer in *Aeromonas salmonicida*, a single surface component may be sufficient to confer hydrophobic and adhesive properties (Trust *et al.*, 1983). However, extensive studies of two Gram-negative microorganisms, *Acinetobacter calcoaceticus* and *Serratia marcescens*, suggest a more complicated general mechanism. Rosenberg and Kjelleberg (1986) have proposed that the degree of hydrophobicity of the microbial cell surface depends on surface components which either promote or reduce cell surface

hydrophobicity (termed, respectively, hydrophobins and hydrophilins). Thus, the amphipathic surface properties of microorganisms are ususally determined by the presence, distribution, configuration and juxtaposition of combinations of hydrophobins and hydrophilins. It is thus not surprising that nonhydrophobic mutants isolated from relatively hydrophobic wild type cells are often deficient in not one, but several surface compoents (Rosenberg *et al.*, 1982; Rosenberg, 1984; Morris *et al.*, 1985).

HYDROPHOBINS AND HYDROPHILINS IN *Acinetobacter calcoaceticus*

In *A. calcoaceticus* RAG-1, thin fimbriae, about 3 nm in diameter, have been implicated in adhesion to and growth on hexadecane (Rosenberg *et al.*, 1982). Mutant strain MR-481, deficient in adhesion to hydrocarbons and polystyrene, was devoid of thin fimbriae. Revertants with an overabundance of thin fimbriae regained partial adhesion properties. However, at least one mutant strain derived from RAG-1, referred to as AB-15, was completely devoid of these fimbriae, and yet adhered to hydrocarbons under certain growth conditions. Moreover, mutant MR-481 cells were found to be devoid of not one, but several surface antigens (M Rosenberg & E A Bayer, unpublished). Thus, there appear to be at least two distinct hydrophobins in this strain.

In *A. calcoaceticus* RAG-1, the presence of a minicapsule, consisting of an anionic heteropolysaccharide, emulsan, has been shown to reduce adhesion to hydrocarbons. This is also true of *A. calcoaceticus* BD-4 cells, which elaborate a rhamnose-containing capsule. Capsulated BD-4 cells are completely incapable of adhering to hexadecane. Enzymatic degradation of the capsule, however, increases adhesion to levels approaching 100% (Rosenberg *et al.*, 1983). Thus, polysaccharide capsule in *A. calcoaceticus* strains appear to function as hydrophilins.

HYDROPHOBINS AND HYDROPHILINS IN *Serratia marcescens*

Adhesion of a pigmented strain of *Serratia marcescens* at the oil:water interface was observed over sixty years ago (Mudd & Mudd, 1924). Many environmental isolates of *Serratia* spp produce an extremely lipophilic tripyrrole pigment, prodigiosin (Ding & Williams, 1983). Spontaneously arising non-pigmented mutants are often deficient in adhering to the air:water interface, as well as to low surface energy surfaces (Hermansson *et al.*, 1979; Rosenberg, 1984). In one study, fourteen clinical isolates of pigmented and nonpigmented *S. marcescens* were compared for adhesion to hydrocarbons and polystyrene (Rosenberg *et al.*, 1986). Whereas pigmented strains adhered best, the nonpigmented strains exhibited moderate to high levels of adhesion to hydrocarbons and polystyrene. Moreover, a nonpigmented mutant of *S. marcescens* RZ exhibited intermediate adhesion to hexadecane and polystyrene, whereas a pigmented but translucent mutant exhibited extremely low adhesion levels (Rosenberg, 1984). Furthermore, 37°C-grown pigmented strains still adhered to hexadecane, even though prodigiosin was not synthesized at that temperature. These data suggested that other component(s), in addition to prodigiosin, act as hydrophobins in these strains. One possible candidate was serratamolide, a cyclic, amphipathic aminolipid (Wasserman *et al.*, 1962), which is found in certain *Serratia marcescens* strains, and is a potent wetting agent (Matsuyama *et al.*, 1985). Serratamolide, however, appears to act as a

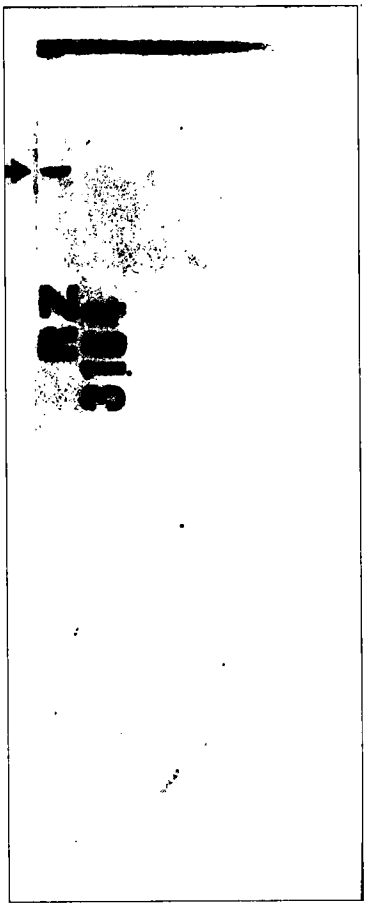

Fig. 1 Western immuno blot analysis of culture supernatants of *S. marcescens* RZ. Antiserum raised in rabbits against whole cells of *S. marcescens* RZ was pre-absorbed with nonhydrophobic mutant 3164 cells. Culture supernatants of wild type RZ (left lane) and mutant 3164 cells (right lane) were electrophoresed on polyacrylamide gel, blotted and reacted with the pre-absorbed antiserum. Reaction of antiserum with the 70 kD serraphobin (arrow) is observed in wild type RZ cells (left lane), but absent in nonhydrophobic mutant 3164 (right lane) which does not produce the 70 kD protein (Bar-Ness & Rosenberg, 1989).

hydrophilin, since wild type serratamolide-synthesizing cells are less hydrophobic than mutants devoid of serratamolide (Bar-Ness *et al.*, 1988). More recently, Bar-Ness and Rosenberg (1989) identified an amphipathic 70 kD surface protein, termed serraphobin, which appears to be the additional hydrophobin of *S. marcescens* RZ. Nonhydrophobic RZ mutant 3164 produces little or no serraphobin (Fig. 1). Furthermore, in wild type RZ cells, serraphobin production and hydrophobicity are lost concomitantly following growth at elevated temperatures. Finally, serraphobin itself is capable of adhering to hydrocarbon droplets. Serraphobin is a predominant outer surface component of RZ cells (Bar-Ness & Rosenberg, 1989), and is a potent immunogen of intact RZ cells (Fig. 1).

Surface structures which have been implicated as hydrophobins or hydrophilins in *A. calcoaceticus* and *S. marcescens* strains are listed in Table 1.

Table 1 Hydrophobins and Hydrophilins in Two Gram Negative Bacteria

Strain	*Component*	*Act(s) as*	*Reference*
Acinetobacter calcoaceticus RAG-1	thin fimbriae	hydrophobins	Rosenberg *et al.*, 1982
A. calcoaceticus RAG-1	emulsan capsule	hydrophilin	Rosenberg *et al.*, 1983
A. calcoaceticus	rhamnose capsule	hydrophilin	Rosenberg *et al.*, 1983
Clinical *Serratia marcescens* isolates and strain RZ	prodigiosin	hydrophobin	Rosenberg *et al.*, 1986; Rosenberg, 1984
S. marcescens RZ	serraphobin (70 kD protein)	hydrophobin	Bar-Ness and Rosenberg, 1989
S. marcescens NS 38	serratamolide	hydrophilin	Bar-Ness *et al.*, 1988

ENHANCEMENT OF MICROBIAL ADHESION TO HYDROCARBONS BY ORGANIC CATIONIC MOLECULES

In general, surfactant molecules tend to inhibit microbial adhesion to hydrocarbons and to other hydrophobic surfaces (Rosenberg & Doyle, 1990). However, recent results have shown that appropriate concentrations of two cationic surfactants, cetylpyridinium chloride and chlorhexidine, can increase adhesion to hexadecane from near 0% to over 90% levels. Mircoorganisms tested included *Escherichia coli*, a nonhydrophobic mutant of *A. calcoaceticus* RAG-1, a poorly adherent strain of *Candida albicans*, and a mixture of microorganisms obtained directly from the oral cavity (Goldberg *et al.*, 1990b). Concentrations of cetylpyridinium chloride which promoted adhesion to hexadecane similarly potentiated adhesion to polystyrene. Similar enhancement of adhesion to hydrocarbons can be demonstrated for larger, cationic polymers, *i.e.* chitosan, poly-L-lysine and lysozyme (Goldberg *et al.*, 1990a). Adhesion mediated by these cationic molecules appears to occur by their adsorption to the cell surface, thus diminishing the negative surface charge and increasing cell surface hydrophobicity.

THE POTENTIAL ROLES OF CELL CONCENTRATION AND DROPLET STABILIZATION

Both *A. calcoaceticus* RAG-1 and *S. marcescens* RZ are strains which, under optimal conditions, adhere to liquid hydrocarbons at levels exceeding 95%. In such cases, adhesion to hydrocarbon appears to be independent of the initial cell concentration employed. For example, adhesion of RAG-1 cells to hexadecane was performed at initial cell concentrations ranging from $4{\cdot}10^4$–$4{\cdot}10^7$. Adhesion was 99·3–99·4%, regardless of initial cell concentration. However, when the kinetic approach was employed,

different removal coefficients were obtained for different initial cell densities (M Barki, unpublished). In *Candida albicans* M7, mixing of low cell concentrations with hexadecane yields droplets which often coalesce within seconds following vortexing, whereas higher cell concentrations yield stable cell-coated droplets and relatively high adhesion levels (Barki & Rosenberg, unpublished). A similar dependence of adhesion levels on increasing cell concentration has been observed with *Escherichia coli* cells which have been hydrophobized by chemical treatment (Goldberg *et al.*, 1990a). One potential explanation for the above phenomena is that the MATH assay is, at least in some cases, dependent on two mechanisms: (i) initial binding of cells at the hydrocarbon surface, and (ii) interactions among the adhering cells at the hydrocarbon:water interface which stabilizies the cell-coated droplets. If this is the case, droplet stability may be dependent on the numbers of adhering cells. In adhesion of *Bacteroides gingivalis* to hexadecane, the cells appear to form a rigid lattice which girdles the droplets; hexadecane areas which are free of bound cells tend to bulge outwards (Rosenberg *et al.*, 1991, submitted). Cooperative interactions among adhering cells have often been ignored in the scientific literature. The possibility that such interactions play a role in the MATH test warrants further investigation.

CONCLUSIONS

Microbial adhesion to hydrocarbons has been employed as a convenient test for microbial hydrophobicity for the past decade. In several strains studied to date, adhesion to hydrocarbons appears to be determined by a complex array of distinct surface structures, which may coexist on the cell surface. Microbial adhesion to hydrocarbons can be potentiated by appropriate concentrations of a range of organic cationic molecules. Recent observations suggest that adhesion to the hydrocarbon droplets depends not only on the initial affinity of the cells for the oil:water interface, but also on the ability of the adhering cells to stabilize the droplets to which they are bound. Thus, while the MATH test has gained popularity because it is so easy to do, the underlying mechanisms may be much more complex than originally assumed.

Acknowledgements

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BACTERIAL DESORPTION BY COMMERCIAL MOUTHWASHES *vs* TWO-PHASE OIL:WATER FORMULATIONS

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During the past eight years, various investigators have shown that oral microorganisms tend to have hydrophobic surface properties. Most oral microorganisms isolated directly from the oral cavity, as well as many oral laboratory strains, adhere to oil droplets. The authors have recently shown that certain concentrations of the cationic antibacterial agents, i.e. cetylpyridinium chloride (CPC) and chlorhexidine (CHX) promote binding of oral and other microorganisms to oil droplets. This has resulted in development of novel aqueous:oil two-phase mouthwashes containing CPC and/or CHX. In the present manuscript, two such formulations were compared with other commercial mouthwashes for their ability to desorb microorganisms from a solid surface (polystyrene). Of the various mouthrinses tested, only the oil:water formulations efficiently removed the adherent bacterial layer. The data suggest that oil:water mouthwashes may have desorption properties superior to those of many commercial mouthwashes.

KEY WORDS: Adhesion, desorption, mouthwash, polystyrene, oil

INTRODUCTION

Bacterial adhesion to oral surfaces is considered a prerequisite for subsequent colonization (Gibbons, 1984). Development of biofilms on soft (mucosal) oral surfaces is kept in check by continual desquamation of epithelial cells, together with their attached microbiota. However, bacterial accumulation on teeth quickly reaches macroscopic proportions (i.e. dental plaque).

One conceivable approach to limiting bacterial film formation in the oral cavity is to inhibit the initial adhesion process. Indeed, addition of certain sugars, surfactants and chaotropic agents can block adhesion of oral microorganisms to model surfaces, and to one another (McIntire *et al.*, 1982; Nesbitt *et al.*, 1982; E Rosenberg *et al.*, 1983). However, such agents are relatively ineffective in removing bacteria which are already attached. Methods of removing bound microorganisms thus appear to have greater potential. Whereas many studies have been dedicated to investigation of the *in vitro* antimicrobial effects of mouthwashes (Perdok *et al.*, 1989), few, if any, reports exist regarding their bacterial desorption properties.

In 1983, desorption of *Acinetobacter calcoaceticus* RAG-1 cells from polystyrene was reported, using simple oil:saline combinations (Rosenberg *et al.*, 1983). The authors have recently presented evidence that addition of certain cationic antibacterial agents (cetylpyridinium chloride [CPC] and chlorhexidine [CHX]) can greatly increase

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adhesion of microorganisms and oral debris to oil droplets (Goldberg *et al.*, 1990). This led to the premise that oil:water mouthrinse formulations containing such agents could serve as effective desorbing mouthrinses, with additional antimicrobial properties.

In the present manuscript, the *in vitro* bacterial desorption ability of two such oil: water formulations is compared with that of commercially available mouthrinses, using a modified *in vitro* technique (Rosenberg *et al.*, 1983).

MATERIALS AND METHODS

Acinetobacter calcoaceticus RAG-1 (ATCC 31012) cells were grown in brain heart infusion broth (Difco Ltd) to stationary phase (18 h) with shaking. Cells were harvested, washed twice and suspended in phosphate buffer (22·2 g $K_2HP0_4 \cdot 3H_2O$, 7·26 g KH_2PO_4 and distilled water to 1 l, pH 7·1).

One ml of a very turbid suspension of RAG-1 cells (O.D. = 9·3, 400 nm) was added to disposable polystyrene cuvettes, 1 cm square (4 ml capacity). Following vortexing for 1 min, the RAG-1 cells were poured off, leaving a film of adherent cells. The cuvettes were washed briefly with tap water. To each cuvette, 1 ml of mouthwash or water (control) was added (Fig. 1) and the cuvettes vortexed for 2 min. The mouthwash was poured off, 1 ml fresh mouthwash was added, and the cuvettes again vortexed for 2 min. The mouthwash was poured off, and the remaining bacterial film stained by adding gentian violet to the cuvettes for 10 min, followed by washing with water to remove excess stain. The remaining bacterial film was quantified by measuring the optical absorbance (585 nm) from both sides of the dried cuvettes in a Uvikon 710 spectrophotometer (Kontron, Zürich, Switzerland).

Results are presented as the average percent decrease in optical absorbance as compared to control cuvettes rinsed with water alone. The types of commercial mouthrinses used in the study, and their origin are presented in Table 1.

Odex is a propriety formulation provided by Agis Ltd consisting of an aqueous solution containing CPC, sweetener, and food colour, and an oil phase (15% v/v) containing a mixture of vegetable and essential oils. Odex+ contains, in addition, 0·025% chlorhexidine gluconate.

RESULTS

In the technique employed, polystyrene cuvettes are first coated with an adherent layer of *Acinetobacter calcoaceticus* RAG-1 cells. Adhesion of this microorganism to surfaces has been extensively studied (Rosenberg & Kjelleberg, 1986), and its avid adhesion to polystyrene has previously been demonstrated (Rosenberg, 1981; Rosenberg *et al.*, 1983). In the original technique, following desorption by oil:water mixtures, outgrowth of RAG-1 cells was used to quantify the residual layer of adherent microorganisms (Rosenberg *et al.*, 1983). Since the present formulations contain antibacterial agents, this approach was modified by using a staining procedure to measure residual adherent cells. This, in turn, entailed employing a much more turbid cell suspension in order to obtain a thick film of adherent microorganisms. Quantitation of desorption could then be ascertained spectrophotometrically.

Results of a typical experiment are summarized in Figure 2 and Table 2. Among the 12 mouthwashes tested, only Odex and Odex+ were highly effective in removing the dense bacterial film, desorbing 98% and 92%, respectively, as compared to the control

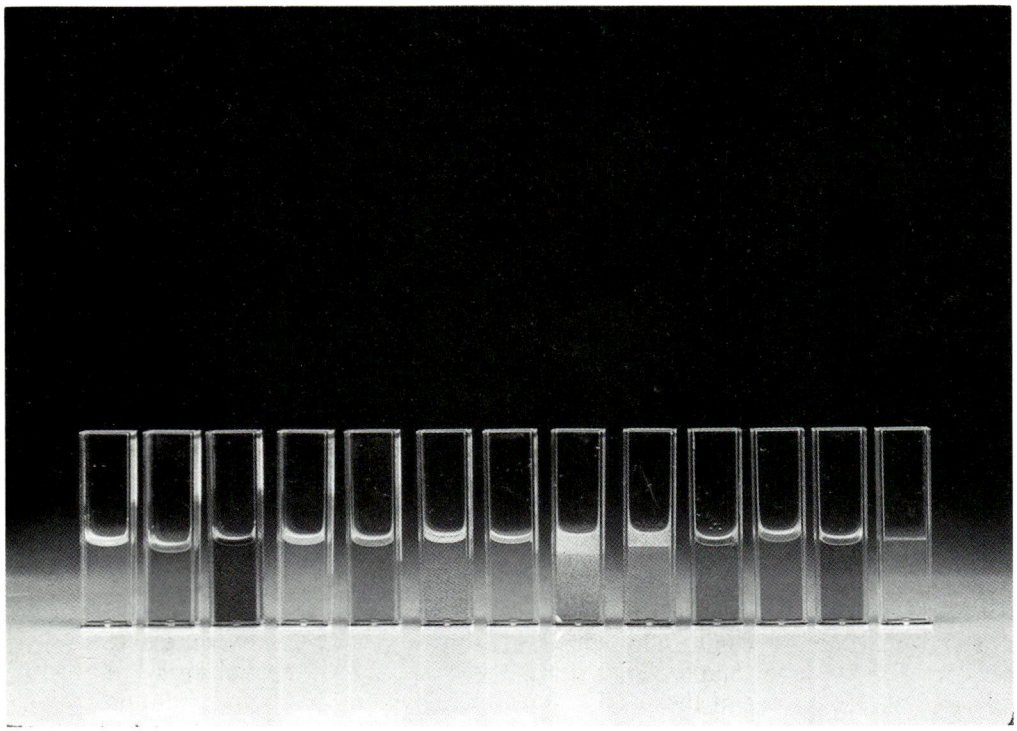

Fig. 1 Polystyrene cuvettes containing the mouthwashes, prior to mixing. From left to right: Listerine®, Eludril, Act®, Cepacol®, Listermint™, Scope®, Corsodyl™, Odex, Odex+, Colgate® Tartar Control Mouthwash, Veadent®, Signal®, water. (See colour plate).

cuvettes rinsed in water alone. Other commercial mouthrinses tested removed between 15% (Corsodyl ™) and 50% (Listerine®) of the bacterial film. Desorption by both oil: water formulations was significantly greater ($P < 0{\cdot}005$) than that observed for all the other mouthwashes.

DISCUSSION

The present report shows that oil:water formulations containing CPC or CPC and CHX, have the capacity to desorb large proportions of bound microorganisms from surfaces as compared to conventional mouthwashes. It has been previously shown (Rosenberg *et al.*, 1983) that aqueous:olive combinations removed over 97% of bacterial film from polystyrene cuvettes, compared to control cuvettes rinsed with water alone. In that study, desorption was measured by subsequent outgrowth of the remaining cell layer. However, since commercial mouthrinses routinely contain antibacterial agents, the outgrowth approach precluded comparison of the desorption efficacy of oil: water mixtures with commercial preparations. Furthermore, a much higher initial concentration of cells was used in the present study, resulting in a more recalcitrant biofilm. Finally, the presence of the cationic antibacterial surface active agents, cetylpyridinium chloride and chlorhexidine, in the oil:water formulations did not

Table 1 Mouthrinses Used in This Study

Name of Mouthwash	*Manufacturer*
Listerine®	Warner-Lambert Co., Morris Plains, NJ 67950
Eludril	PF Medicament, 125, rue de la Faisanderie, Paris, France
Act®	Johnson & Johnson, New Brunswick, NJ 08903
Cepacol®	Merrell Dow Pharmaceuticals Inc, Cincinnati, Ohio 45215
Listermint (trade mark)	Warner Lambert Health Care, Eastleigh, Hants, England
Scope® (Peppermint)	Proctor & Gamble, Cincinnati, Ohio 54202
Corsodyl (trademark)	Imperial Chemical Industries, PLC, Macclesfield, Cheshire, Great Britain
Odex	Agis Ltd, Tel-Aviv
Odex +	Agis Ltd, Tel-Aviv
Colgate® Tartar Control Mouthwash	Colgate Palmolive Co., NY, NY 10022
Veadent®	Glaxo (under license from Vipont Laboratories Inc), Fl. Collins, USA
Signal®	Lever Brothers Co., 390 Park Avenue, NY, NY 10022

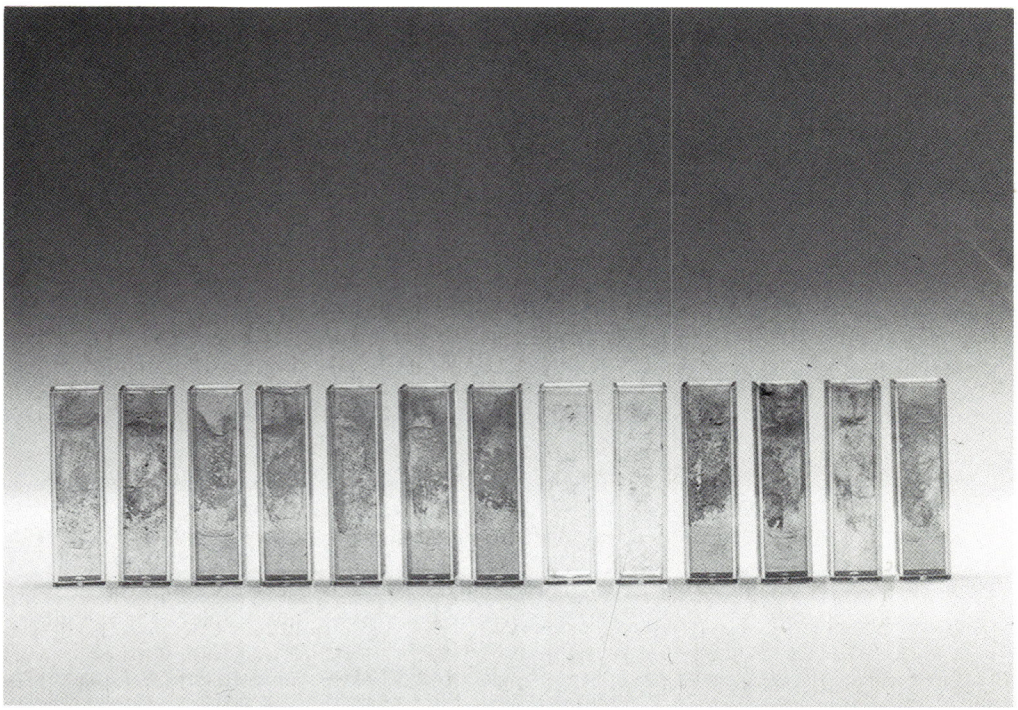

Fig. 2 Stained residual bacterial layer, following rinsing procedure. Cuvettes were layered with bacterial film, washed, rinsed with mouthwashes and stained as described in Materials and Methods. Order of cuvettes is the same as Figure 1. (See colour plate).

Table 2 Desorption of an Adherent Bacterial Film by Mouthwashes

Name of Mouthwash	*Percent Desorption compared to water control*[a]
Listerine®	50
Eludril	34
Act®	40
Cepacol®	40
Listermint (trademark)	36
Scope® (Peppermint)	28
Corsodyl (trademark)	15
Odex	98
Odex+	92
Colgate® Tartar Control Mouthwash	21
Veadent®	28
Signal	44

[a]Average of 2 readings

adversely affect the desorption properties. Addition of either of these agents to oil: water mixtures results in long-term reductions in oral malodour and microbial levels (Y Konis & M Rosenberg, unpublished). It should be emphasized that commercial mouthrinses containing cetylpyridinium chloride (e.g. Cepacol®, Scope®), or chlorhexidine (e.g. Eludril, Corsodyl (trademark)) were relatively ineffective in desorbing the bacterial layer. Indeed, Odex preparations are relatively ineffective desorption agents when the oil phase is excluded.

The present study is based on an *in vitro* technique based on desorption from a smooth, hydrophobic surface. Whereas hydrophobic interactions are important for oral adhesion, they probably act in concert with other mechanisms. Nevertheless, hydrophobic inhibitors are able to block adhesion to oral surfaces and to desorb adherent cells (Nesbitt *et al.*, 1982; Rosenberg and Kjelleberg, 1986; E Rosenberg *et al.*, 1983). Moreover, whereas the well-characterized model organism employed here (RAG-1) is not an oral microorganism, ODEX also effectively desorbs *Actinomyces viscosus* and *Streptococcus sanguis* from polystyrene tubes (not shown). Furthermore, microscopic observation of ODEX following intraoral use shows that high levels of oral microorganisms are concentrated on the surface of expectorated oil droplets (Rosenberg, 1990*b*).

Finally, *in vitro* experiments indicate that rinsing with ODEX removes more stainable oral debris than rinsing with the aqueous phase of ODEX alone. These findings suggest that ODEX also has *in vivo* oral desorption capabilities and that the oil phase is critical for the desorbing effect.

The above notwithstanding, it is likely that efficient bacterial desorption is only one of several criteria to be considered when discussing mouthwash efficacy. For example, whereas Corsodyl™, a chlorhexidine mouthrinse, is highly effective in reduction of microbial levels (Perdok *et al.*, 1989) and oral malodour (Rosenberg, 1990*a*), in the

present report, it exhibited the poorest desorption capacity among all mouthwashes tested. We can consider the following hypothetical example: a nonbacteriostatic, nonbactericidal mouthwash that desorbs 75% of oral microorganisms (a daunting challenge, considering the concentration of oral microorganisms in multilayered plaque and sequestered subgingival sites) can be effective for only two generations, since only two doublings are required to regain original microbial levels. However, a mouthwash that desorbs 75% of the oral microbiota and kills 75% of the residual microorganisms would be three times as effective. Thus, a formulation such as ODEX+, which is still an effective desorber (92%), yet contains a cocktail of antibacterials, including CHX, may have high efficacy. *In vivo* experiments to test the long-range effects of such oil:water formulations on oral microbial levels and general oral hygiene and health are thus warranted.

Acknowledgement

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Colour Plate 1 (See Goldberg & Rosenberg "Bacterial Desorption by Mouthwashes . . .")

Fig. 1 Polystyrene cuvettes containing the mouthwashes, prior to mixing. From left to right: Listerine®, Eludril, Act®, Cepacol®, Listermint™, Scope®, Corsodyl™, Odex, Odex+, Colgate® Tartar Control Mouthwash, Veadent®, Signal®, water.

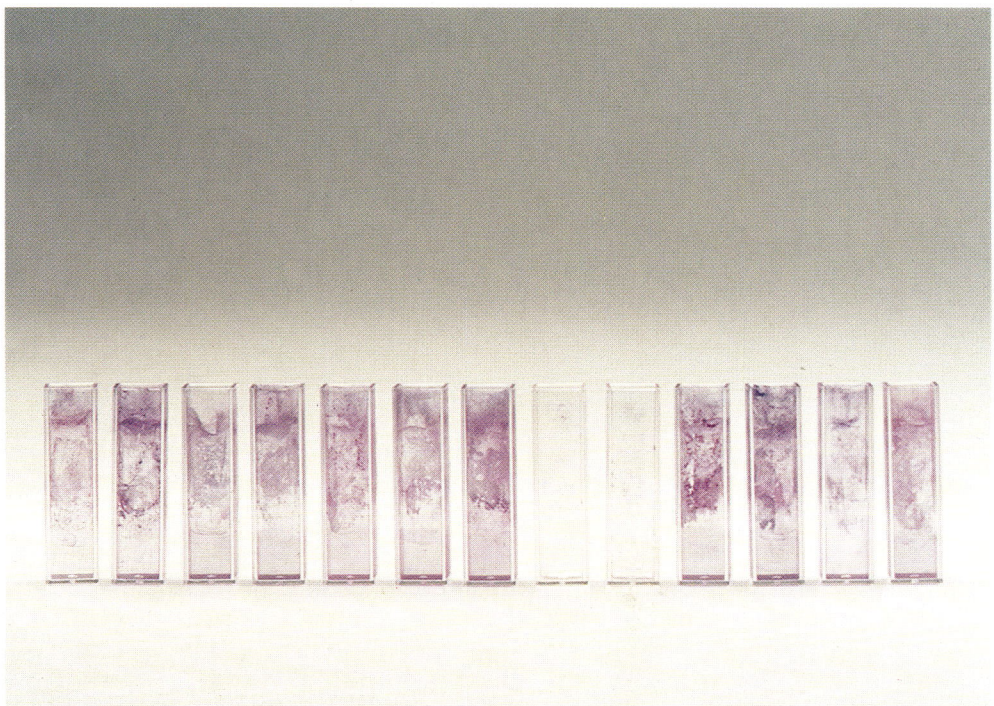

Fig. 2 Stained residual bacterial layer, following rinsing procedure. Cuvettes were layered with bacterial film, washed, rinsed with mouthwashes and stained as described in Materials and Methods. Order of cuvettes is the same as Figure 1.

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Articles (42)

Adhesion of *Actinomyces viscosus* to *Porphyromonas (Bacteroides) gingivalis*-Coated Hexadecane Droplets

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Interbacterial adhesion (coadhesion) is considered a major determinant of dental plaque ecology. In this report, we studied several aspects of the adhesion of *Porphyromonas (Bacteroides) gingivalis* to hexadecane in order to use the liquid hydrocarbon as a convenient substratum for coadhesion assays. Washed suspensions of hydrophobic *P. gingivalis* 2561 cells were vortexed with hexadecane to yield highly stable cell-coated droplets. Kinetics of coadhesion between *Actinomyces viscosus* cells and *P. gingivalis*-coated hexadecane droplets (PCHD) was subsequently studied. Aliquots of PCHD were added to *A. viscosus* suspensions, and the mixtures were gently rotated. Avid adhesion of *A. viscosus* cells to the immobilized *P. gingivalis* layer could be readily measured by the decrease in turbidity in the aqueous phase, following phase separation. Despite the ability of *A. viscosus* cells to adsorb to hexadecane following vigorous mixing, gentle mixing did not appreciably promote adhesion to bare hexadecane. Moreover, extensive microscopic examinations revealed that *A. viscosus* cells adhered exclusively to the bound *P. gingivalis* cells rather than to exposed areas of hexadecane. Coadhesion of *A. viscosus* to the PCHD appeared to follow first-order kinetics, attaining 80% levels within 30 min. Electron micrographs revealed *A. viscosus* cells adhering to the *P. gingivalis* cell layer adsorbed at the hexadecane-water interface. Interestingly, *P. gingivalis* cells did not appear to penetrate the hexadecane. *A. viscosus* mutants lacking type 1 or type 2 fimbriae or both were still able to bind to the PCHD. No obvious correlation was observed between relative hydrophobicity of *A. viscosus* strains and their binding to PCHD. However, defatted bovine serum albumin, an inhibitor of hydrophobic interactions, was the most potent inhibitor among those tested. The data suggest that this approach provides a simple, quantitative technique for studying kinetics of bacterial coadhesion which is amenable to both light and electron microscopic observation.

Surfaces submerged in an aqueous environment are prone to rapid colonization by adhering microbial populations. Initial microbial adsorption may be followed by adhesion of additional microorganisms from the bulk aqueous phase to the developing microbial layer. The buildup of adherent macroscopic masses of attached microbiota requires intimate cell-cell interactions which protect the community from being dislodged. Such biofilms are common in the oral cavity, particularly on tooth surfaces (dental plaque). In recent years, the role of interbacterial adhesion (coadhesion) in dental plaque ecology has drawn increasing interest (15). Whereas adhesion to freshly cleaned tooth surfaces occurs rapidly, the majority of microorganisms in maturing dental plaque are bound to one another rather than to the salivary pellicle on the tooth surface (5, 7, 15). Certain bacteria may adhere preferentially to preformed plaque rather than directly to the tooth surface (5). Finally, coadhesion between interdependent species in dental plaque may promote synergistic physiological relations (7, 17).

Interbacterial adhesion of oral species was first demonstrated by Gibbons and Nygaard (12). Indeed, most studies to date have employed so-called coaggregation assays, in which aqueous suspensions of two strains are mixed and the degree of association is determined visually (degree of clumping) or by quantitative means (6, 7, 16, 18). This approach, while simple to perform, is difficult to observe at the electron microscope level without altering cell-cell orientation or distance. The contributions of intrastrain aggregation (i.e., autoaggregation) are hard to assess in such assays. Moreover, adhesion of cells to preformed layers may be a general natural phenomenon in biofilm accumulation, whereas the ecological relevance of in vitro coaggregation of cells is not intuitively clear. Thus, several researchers have recently proposed studying coadhesion of one strain, free in suspension, to a preformed layer of the second strain adhering on a given surface. For example, Liljemark et al. used a continuous layer of *Streptococcus sanguis* cells affixed to plastic and enamel surfaces with a commercial adhesive (Cell-TAK) to study coadhesion with *Haemophilus parainfluenzae* and *Streptococcus sobrinus* (17). Ellen and coworkers have studied attachment of *Bacteroides gingivalis* to preformed layers of *Actinomyces viscosus* strains on saliva-coated hydroxyapatite beads (7, 27). The technical complexities of preparing the substratum, coating it with the first species, and measuring coadhesion of the second species prompted us to search for a simpler solution which would also be amenable to light and electron microscopy. This was carried out by (i) immobilizing *Porphyromonas (Bacteroides) gingivalis* cells on hexadecane droplets by a simple mixing procedure and (ii) following coadhesion of free *A. viscosus* cells to the *P. gingivalis*-coated droplets as a function of time.

MATERIALS AND METHODS

***A. viscosus* strains and culture conditions.** *A. viscosus* WVU627 was originally obtained from M. A. Gerencser, West Virginia University. Strain T14VJ1 (bearing both type 1 and type 2 fimbriae) and corresponding mutant strains 5519

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(lacking type 2 fimbriae), 5951 (lacking type 1 fimbriae), and 147 (lacking both fimbrial types) were obtained from J. Cisar, National Institute of Dental Research. These strains have been reported to differ in their adhesion properties (2). Strains were maintained on brain heart infusion agar slants (Difco Ltd.) and stored at 4°C. *A. viscosus* strains were inoculated from slants into test tubes containing 10 ml of tryptic soy broth (Difco Ltd.) and grown for 48 h at 37°C under anaerobic conditions (80% N_2, 10% CO_2, 10% H_2). For adhesion experiments, 1:1,000 dilutions were inoculated into test tubes containing the same medium and grown for 48 h as described above. Cells were harvested by low-speed centrifugation, washed three times in phosphate (P) buffer (7.26 g of KH_2PO_4, 22 g of $K_2HPO_4 \cdot 3H_2O$, distilled water to 1,000 ml [pH 7.1]), and suspended in buffer in glass test tubes (10-mm inner diameter) to obtain optical densities (corrected for nonlinearity) from ca. 1.0 to 2.0, as measured at 550 nm in a model 350 spectrophotometer (G.K. Turner Associates, Palo Alto, Calif.).

***P. gingivalis* strains and culture conditions.** *P. gingivalis* 2561 (ATCC 33277, type strain) was obtained from J. Slots, University of Pennsylvania. *P. gingivalis* W50 was obtained from J. Zambon, State University of New York at Buffalo. *P. gingivalis* strains were maintained on laked blood agar, comprising blood agar base no. 2 (Oxoid Ltd., Basingstoke, Hampshire, England) supplemented with 7% laked sheep blood and 1 mg each of filter-sterilized hemin and menadione per ml. Cells were inoculated into a modified Trypticase-peptone-yeast extract broth containing Trypticase-peptone (BBL Microbiology Systems, Cockeysville, Md.) supplemented with yeast extract (Difco Ltd.) (3 g), NaCl (5 g), K_2HPO_4 (2.5 g), glucose (2.5 g), filter-sterilized hemin (5 mg), filter-sterilized menadione (0.5 mg), and $NaHCO_3$ (1 g) (11). All cultures were incubated at 37°C in anaerobic jars containing 80% N_2, 10% CO_2, and 10% H_2. Following 72 h of growth, cells were harvested and washed four times in P buffer. The cell suspension was passed once through a 25-gauge needle to more evenly suspend the cells and adjusted to a corrected optical density of ca. 2.0.

Preparation of *P. gingivalis*-coated hexadecane droplets (PCHD). *P. gingivalis* cell suspensions (optical density, 2.0), prepared as described above, were distributed in 2.0-ml aliquots into clean borosilicate glass test tubes. *n*-Hexadecane (0.15 ml) was added to each tube. The test tubes were then vortexed at the highest speed setting for four consecutive 1-min periods on a Vortex-Genie (Scientific Industries, Inc., Bohemia, N.Y.). After phase separation, the percent adhesion was determined by the decrease in turbidity of the lower aqueous phase. The contents of the test tubes were then pooled into a beaker, and the lower aqueous phase, containing unbound cells, was carefully removed by using a Pasteur pipette. For every milliliter of lower aqueous phase extracted, 0.6 ml of fresh P buffer was added. The suspension was swirled gently prior to dispensing into test tubes for the coadhesion assay to ensure uniform distribution of the droplets. *A. viscosus*-coated hexadecane droplets were prepared in a similar manner, by employing strain WVU627.

Coadhesion assay. After several preliminary experiments, the following protocol was adopted. To 1.0 ml of *A. viscosus* cell suspension in clean borosilicate glass test tubes (13-mm outer diameter; 11-mm inner diameter; 100-mm length) were added 0.5 ml of P buffer, with or without inhibitors, and 0.5 ml of mixed PCHD suspension. Controls included (i) test tubes containing hexadecane (62.5 μl) and 937.5 μl of P buffer rather than PCHD, (ii) test tubes containing only *A. viscosus* cell suspension in P buffer, with or without inhibitors, as indicated, and (iii) test tubes containing only PCHD and P buffer, with or without inhibitors, as indicated. All test tubes were incubated with gentle rotation at a fixed incline of 40° in a Multi-purpose Rotator (Scientific Industries, Inc., Springfield, Mass). At 2-min intervals, phases were allowed to separate and turbidity of the lower aqueous phase was measured directly in the spectrophotometer. Experiments were repeated at least two times; representative data are shown. The degree of coadhesion was calculated as the percent decrease in turbidity of the aqueous phase, compared with the initial reading. Photomicrographs of PCHD with and without adherent *A. viscosus* cells, stained with gentian violet (BDH Inc., Toronto, Ontario, Canada), were taken in a Leitz Orthoplan light microscope (Leitz Wetzlar, Wetzlar, Federal Republic of Germany).

Hydrophobicity measurements of *A. viscosus*. Bacterial adhesion to hexadecane has been described previously (23–26). Washed *A. viscosus* cell suspensions (optical densities, 1 to 2) in P buffer were distributed in 2.0-ml aliquots into clean borosilicate glass test tubes. *n*-Hexadecane (0.15 ml) was added to each tube. The test tubes were then vortexed as described above for *P. gingivalis*. The decrease in optical absorbance of the lower aqueous phase was measured as a function of mixing time. Adhesion to polystyrene microtiter plate wells (non-tissue culture treated) of 100-μl aliquots of the washed *A. viscosus* cell suspensions was performed for 20 min as previously described (23).

Electron microscopy. Aliquots (50 μl) containing suspensions of bacteria-coated hexadecane droplets, with or without coadhering strains, were mixed with 200 μl of molten (50°C) 1% Noble agar (Difco Ltd.) and allowed to solidify at room temperature within Pasteur pipettes. After the solidified samples were ejected onto clean glass slides, they were cut into segments of 5 to 7 mm in length. The segments were subsequently prefixed in 0.1 M sodium phosphate buffer, pH 7.4, containing 1% glutaraldehyde for 1 h, which was followed by two washes and overnight incubation in P buffer. Postfixation was carried out in 2% OsO_4 in sodium phosphate buffer. Following three washes in P buffer (pH 7.1), the segments were dehydrated in graded ethanol solutions and then washed twice in propylene oxide (Ernest F. Fullam, Inc., Schenectady, N.Y.). The segments were then embedded in Epox 812 resin (Ernest F. Fullam, Inc.). Thin sections were cut and transferred to single-slot, Formvar-coated nickel grids. Following staining with 5% uranyl acetate and Reynold's lead citrate, the samples were examined and photographed by using a Phillips 400T transmission electron microscope.

Chemicals. Except when otherwise noted, chemicals and biochemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.).

RESULTS

Preliminary experiments were carried out to determine whether bacteria-coated hexadecane droplets could be used as a substratum for coadhesion studies. With the exception of *P. gingivalis* W50, the strains studied here all adhered to hexadecane in high proportions (Fig. 1). Similarly, adhesion to a hydrophobic solid surface, polystyrene, was evident for all strains which adhered well to hexadecane (data not shown). We initially envisioned measuring binding of free *P. gingivalis* cells to *A. viscosus* WVU627-coated hexadecane droplets. However, this process was difficult to follow quantitatively, since the free *P. gingivalis* cells in suspension tended to autoaggregate. Thus, *P. gingivalis*-coated hexa-

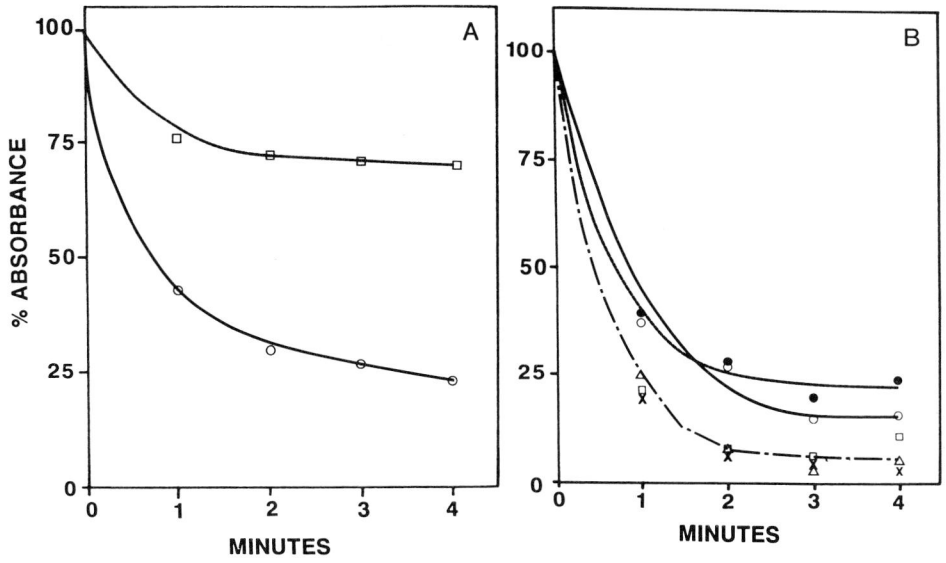

FIG. 1. (A) Adhesion of *P. gingivalis* to hexadecane. Adhesion of *P. gingivalis* 2561 (○) and *P. gingivalis* W50 (□) to hexadecane was carried out as described in Materials and Methods. Results are presented as percent absorbance in the lower aqueous phase as a function of mixing time. (B) Adhesion of *A. viscosus* strains to hexadecane. Adhesion of *A. viscosus* WVU627 (●), T14VJ1 (X), 5951 (□), 5519 (△), and 147 (○) to hexadecane.

decane droplets were subsequently studied as a substratum for coadhesion with *A. viscosus* cell suspensions.

P. gingivalis 2561 routinely adhered to hexadecane at levels above 80% (Fig. 1A). PCHD were stable for several days at room temperature. Light microscopic observation of cell-coated droplets was performed by using gentian violet to visualize the bound cells. In many cases, droplets were observed which were completely covered by a monolayer of adherent cells. Other droplets, however, were covered by patches or rafts of associated cells (Fig. 2a). Some droplets developed protrusions, in which the round contour of the droplet was maintained in regions covered by cells, but bare areas of hydrocarbon could be observed extruding outwards from the droplets (Fig. 2b). In contrast, vortexing hexadecane with the much less hydrophobic *P. gingivalis* W50 yielded unstable droplets which tended to coalesce rapidly, and these droplets bore only a few widely spaced cells when viewed by microscopy.

Adhesion of *P. gingivalis* 2561 to hexadecane was also observed by using electron microscopy (Fig. 3). In addition to bound cells, free vesicles, probably released during growth, washing, and dispersion procedures, could be observed. Of considerable interest was the observation that the cells did not appear to penetrate into the hexadecane phase or to otherwise deform the interface.

Since all the *A. viscosus* strains tested adhered to hexadecane following vigorous mixing (Fig. 1B), conditions were sought which would promote coadhesion to the bound *P. gingivalis* cells rather than adsorption directly to the hexadecane substratum. This problem was overcome by gentle mixing in tilted test tubes. Under such conditions, *A. viscosus* did not adhere appreciably to hexadecane, yet avid coadhesion of *A. viscosus* cells free in suspension to the bound *P. gingivalis* cells on the hexadecane droplets was observed (Fig. 4). Moreover, microscopic observation of hundreds of droplets confirmed that *A. viscosus* cells adhered exclusively to bound *P. gingivalis* cells and not to exposed areas of the hexadecane (Fig. 5). Coadhesion of *A. viscosus* cells to the *P. gingivalis* cell layer was further corroborated by observation of thin sections in the electron microscope (Fig. 6). As an additional control of specificity, coadhesion of *A. viscosus* to *S. sanguis*-coated hexadecane droplets was also studied under the same conditions. In the calcium-free buffer employed in the present study, coadhesion was not observed. Finally, the ability of *P. gingivalis* cells to coaggregate when mixed with *A. viscosus* on glass slides remained intact following their (*P. gingivalis*) desorption from the hexadecane by solidification of the hexadecane phase (26).

The kinetics of coadhesion was followed by turbidimetric measurement of the unbound *A. viscosus* cell suspension in the lower aqueous phase, as a function of mixing time (Fig. 7). In the presence of PCHD, coadhesion was rapid, attaining 80% levels within 30 min. No significant decrease in turbidity occurred in the control tube containing *A. viscosus* and hexadecane. Similarly, no increase in turbidity (which would suggest desorption of *P. gingivalis* cells) was observed in control tubes containing PCHD without *A. viscosus* (Fig. 7). Coadhesion of *A. viscosus* to PCHD followed apparent first-order kinetics.

The rate of coadhesion depended on the concentration of PCHD in the assay. When one-half of the PCHD mixture was added, the coadhesion rate was approximately half that of the tube containing the higher concentration. As expected from the apparent first-order kinetics, the initial concentration of *A. viscosus* cells did not substantially alter the fraction of cells coadhering as a function of time. The rate of coadhesion was also increased by 73% when double the concentration of *P. gingivalis* was employed to prepare the PCHD, keeping the hexadecane volume constant. In contrast, a much smaller increase (24%) was observed when double the volume of hexadecane was used to form the PCHD but the *P. gingivalis* concentration was kept constant. This latter increase may be due to the adhesion of higher numbers of *P. gingivalis* cells to the higher hexadecane volume or alternatively to a more spacious distribution

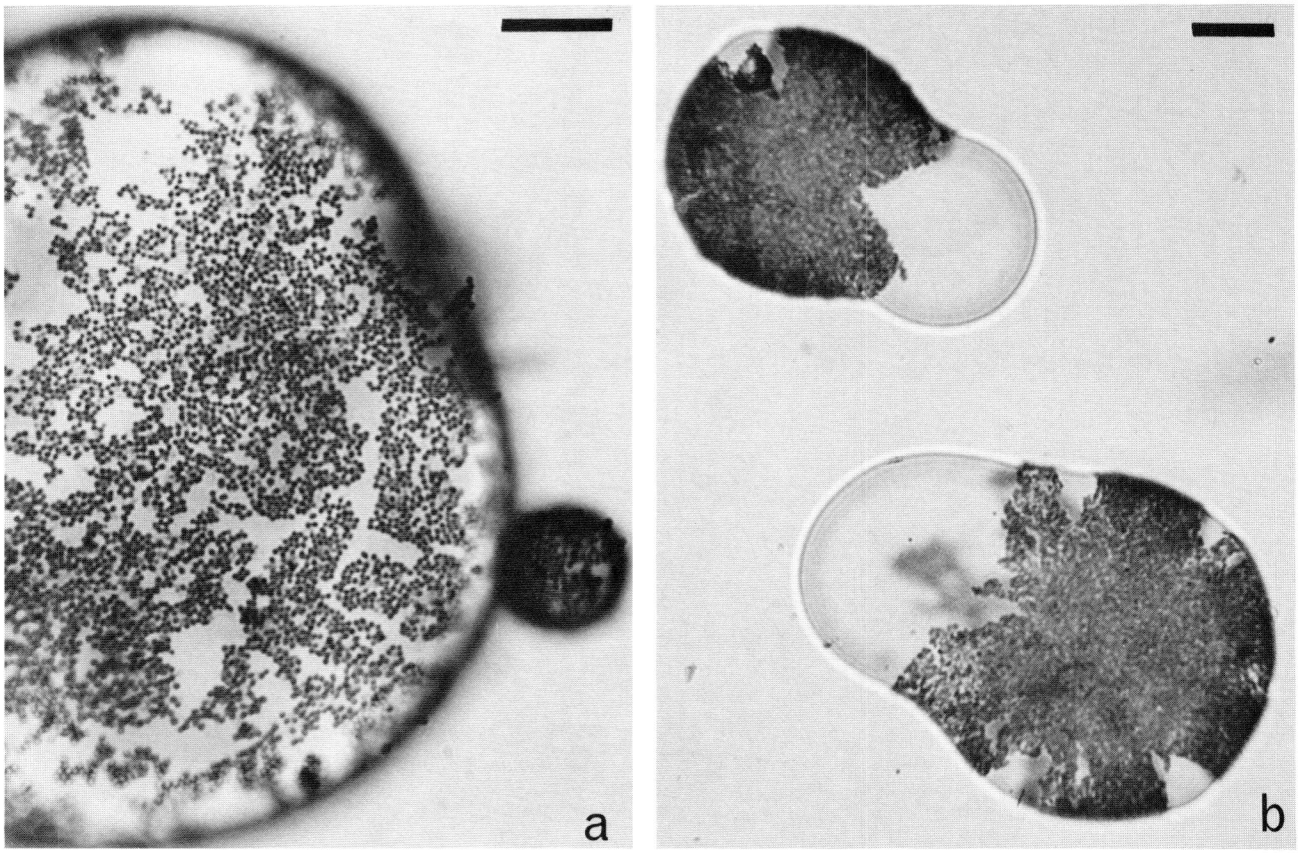

FIG. 2. Light microscopic observation of adhesion of *P. gingivalis* to hexadecane. After adhesion to hexadecane and phase separation, samples of the upper phase were observed by using gentian violet to enhance visualization. (a) Droplet with patches of adherent cells; (b) extrusion of hexadecane from two adjacent droplets. Bars, 10 μm.

of *P. gingivalis* cells. In all these instances, when altering either the concentrations or the volumes used, the straight-line linearity of the plotted curves was maintained.

Since *P. gingivalis* cell suspensions with an optical density of 1.0 correspond to approximately 3.8×10^9 cells per ml, as opposed to approximately 1.3×10^9 cells per ml for the larger actinomyces cells (12a), the *P. gingivalis*/*A. viscosus* ratio in the coadhesion assay could be calculated to be ca. 10:1. Viability of either species was not directly a factor in the assay. *P. gingivalis* cells were no longer viable prior to mixing with hexadecane, as determined by plating of cell suspensions. Heat-killed *A. viscosus* cells (75°C, 30 min) coadhered in higher proportions to the PCHD than cells that were not killed; heat-treated *P. gingivalis* cells (75°C, 30 min) concomitantly lost the ability to adhere to hexadecane, to coaggregate with *A. viscosus*, and to autoaggregate (data not shown).

Coadhesion was studied by employing *A. viscosus* T14VJ1 and its corresponding mutants lacking type 1 fimbriae, type 2 fimbriae, or both (Fig. 8). Both *A. viscosus* mutants devoid of type 2 fimbriae underwent spontaneous aggregation. Nevertheless, the rapid drop in turbidity in the presence of PCHD, compared with the control, as well as microscopic observation of the droplets, demonstrated that extensive coadhesion occurred. Coadhesion rates and hydrophobicity results are compared in Table 1. No correlations were evident between fimbrial type, rate of coadhesion, and extent of adhesion to hexadecane. For example, whereas *A. viscosus* WVU627 adhered less avidly to hexadecane than T14VJ1 did (76% and 97%, respectively), it coadhered with *P. gingivalis* at a much greater rate. Similarly, coadhesion of *A. viscosus* fimbrial mutants could not be correlated with either fimbrial type or hydrophobicity.

Inhibition experiments were carried out by adding potential inhibitors to the aqueous phase prior to the assay. Among the potential inhibitors tested, defatted bovine serum albumin (BSA) was most potent (Fig. 9). For example, 4 mg of defatted BSA per ml inhibited adhesion by 77% following a 14-min incubation. Nondefatted BSA was also inhibitory, but much less so than the defatted protein (only 40% following a 14-min incubation). In the presence of BSA, coadhesion did not appear to follow first-order kinetics (Fig. 9). Previous studies have suggested that L-arginine, among a series of amino acids tested, may inhibit coaggregation of *A. viscosus* and *P. gingivalis* (16a). Under the assay conditions used in these experiments, coadhesion was not significantly inhibited by a variety of amino acids at final concentrations of 10 mM (L-arginine, L-asparagine, L-lysine, or L-phenylalanine [data not shown]). Tryptophan has been shown to inhibit hemagglutination by *Pseudomonas aeruginosa* at a 1 mM concentration (8). However, even 5 mM tryptophan (final concentration) was not significantly inhibitory in the present assay.

DISCUSSION

Bacterial adhesion to hydrocarbons has been extensively applied in studying hydrophobic surface properties of oral

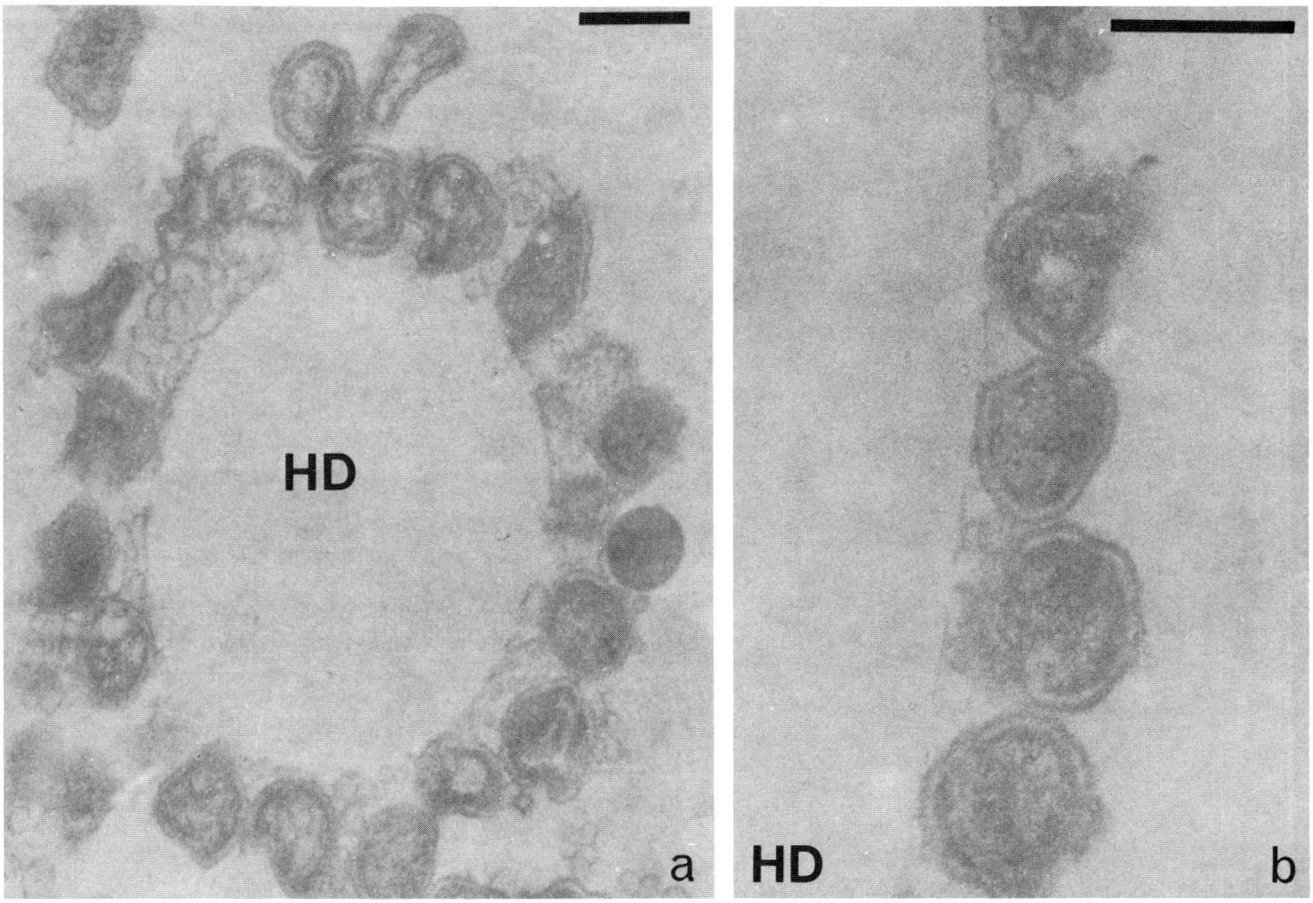

FIG. 3. Electron microscopic observation of adhesion of *P. gingivalis* to hexadecane. Ultrathin sections of cells adhering to hexadecane droplets, stabilized in agar and stained with uranyl acetate and lead citrate, were prepared as described in Materials and Methods. A small hexadecane droplet encompassed by adhering cells is shown (a). No deformation of the interface by the adhering cells is evident (b). Bars, 500 nm.

microorganisms (26). Most freshly obtained oral isolates (29), as well as laboratory strains of streptococci and other species (3, 9, 10, 20, 21, 28), are able to adhere directly to oil droplets after brief mixing periods. In the present report, we have introduced a modification of this technique to study interspecies coadhesion. This method has two stages: (i) adhesion of one of the species to hexadecane droplets under vigorous mixing conditions and (ii) measurement of coadhesion of the second species to the bound cells of the first under very mild mixing conditions. In contrast to a previous report on *Bacteroides* spp. from another laboratory (13), the first stage produced stable PCHD. Microscopic examinations of *P. gingivalis* 2561 cells adhering to the hexadecane droplets yielded several novel observations. In many cases, *P. gingivalis* cells adhered to hexadecane in patches. These resembled the patches of adherent oil-degrading microorganisms which are frequently observed during hydrocarbon fermentations (22a). Under microscopic examination, some droplets developed gross deformations. Hexadecane could be observed extruding outwards from the droplets in regions free of bound cells. Conversely, areas coated with a monolayer of bound cells preserved their circular form. Such deformations, probably resulting from application of pressure on the droplets sandwiched between the microscope slide and coverslip, indicate that the adherent cells act to stabilize the hydrocarbon droplets. Indeed, as has previously been observed with other bacterial strains (23, 24, 26), PCHD could be stored in test tubes at room temperature for days to weeks, while bare hexadecane droplets coalesce almost immediately. It is tempting to hypothesize that this stabilizing effect is due at least in part to attractive, lateral interactions among the adhering cells. Hexadecane droplets were not stabilized by *P. gingivalis* W50, which evidently failed to adhere to hexadecane in numbers high enough to establish cell-cell contact. Adhesion of *P. gingivalis* to hexadecane was also observed by using electron microscopy. To our knowledge, this is the first electron microscopic demonstration of microbial partitioning at the aqueous-hydrocarbon interface. Of considerable interest was the observation that the adhering cells did not appear to penetrate into the hexadecane phase or to substantially deform the interface (Fig. 3), in contrast to the prediction of Mudd and Mudd, which was based on interfacial tension considerations (19).

After adhesion of *P. gingivalis* 2561 to hexadecane, the bottom aqueous phase, containing low levels of unbound cells, was replaced with fresh buffer. Suspensions of PCHD could be readily distributed into test tubes containing suspensions of *A. viscosus* cells. Since coadhering *A. viscosus* cells rose with the PCHD, the rate of coadhesion could be followed directly in the test tubes by turbidimetric readings over time. Coadhesion of *A. viscosus* to *P. gingivalis* cells followed apparent first-order kinetics, i.e., a linear correlation between the rate of coadhesion and the relative concen-

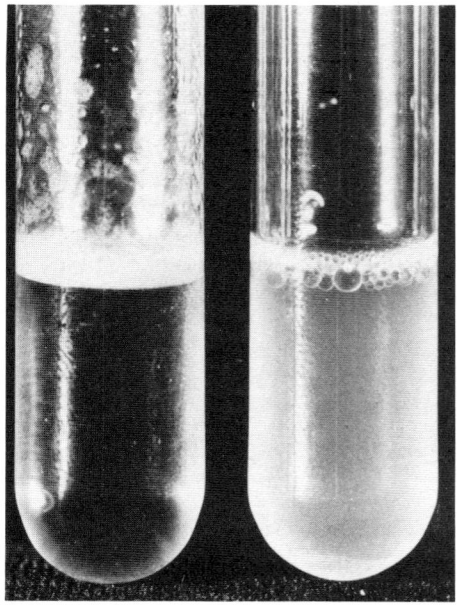

FIG. 4. Coadhesion assay. After 30 min of incubation of *A. viscosus* cells with PCHD, *A. viscosus*-mediated turbidity in the lower aqueous phase disappeared concomitant with coadhesion of cells to PCHD (test tube on left). No significant decrease in turbidity was observed in the control tube (right), which contained *A. viscosus* cell suspension and hexadecane, under the mild mixing conditions employed.

tration of unbound *A. viscosus* cells at any given time. The possibility that adhesion of *A. viscosus* cells to exposed hexadecane was responsible for the observed phenomenon can be ruled out since (i) extensive microscopic observation revealed *A. viscosus* cells adhering exclusively to bound *P. gingivalis* cells rather than to exposed areas of hexadecane, (ii) turbidity of control tubes containing *A. viscosus* cells and hexadecane remained almost constant throughout the experiment, and (iii) there was no correlation between adhesion to hexadecane and coadhesion to PCHD in the five *A. viscosus* strains tested. Furthermore, in previous coadhesion studies using *A. viscosus*-coated hydroxyapatite beads, free *P. gingivalis* cells appeared to bind almost exclusively to *A. viscosus* rather than in gaps between bound *A. viscosus* cells (27). However, the single confirmatory method of scanning electron microscopy in that study left a degree of uncertainty, which is now resolved in the current investigation.

Hydrophobicity per se did not appear to be the critical factor in the coadhesion observed. Although all the *A. viscosus* strains tested adhered to hexadecane and polystyrene, no obvious correlations were observed between hydrophobicity and coadhesion. Furthermore, microscopic observations clearly indicated that under the mild mixing conditions employed, *A. viscosus* cells adhered preferentially to *P. gingivalis* cells rather than to the hexadecane surface. Nevertheless, the participation of hydrophobic interactions cannot be conclusively ruled out by the data.

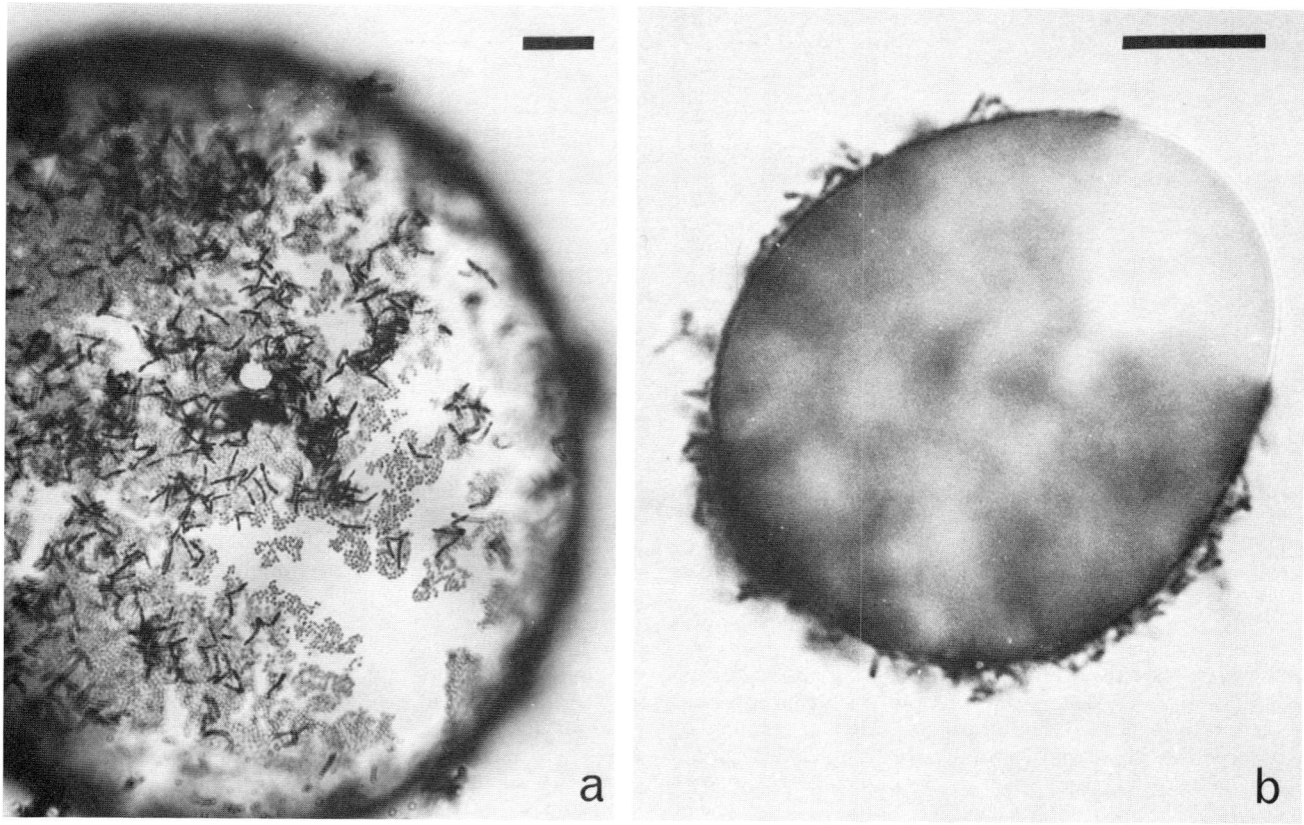

FIG. 5. Adhesion of *A. viscosus* WVU627 to PCHD. Incubation of *A. viscosus* cells with PCHD was carried out as described in Materials and Methods. Following a 30-min incubation, the upper phase was removed and examined by light microscopy after being stained with gentian violet. (a) *A. viscosus* cells (dark rods) can be observed coadhering to patches of *P. gingivalis* cells (lighter coccal cells) on a hexadecane droplet. (b) *A. viscosus* cells can be seen coadhering to the *P. gingivalis* cell layer around the perimeter of the hydrocarbon droplet. Bars, 10 μm.

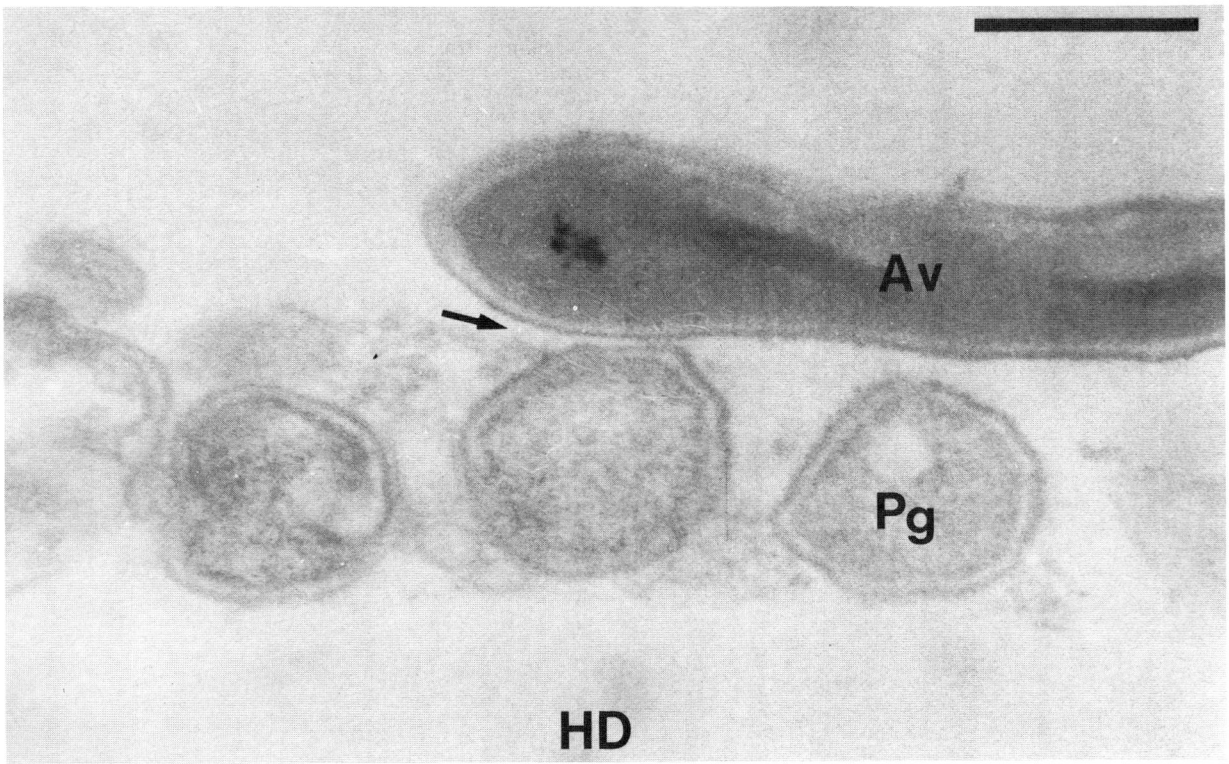

FIG. 6. Electron micrograph of coadhesion. Coadhesion of *A. viscosus* cell (Av) (longer cell) to *P. gingivalis* cells (Pg) adhering to hexadecane droplet (HD). Note the apparent involvement of a vesicle (bleb) associated with *P. gingivalis* cells (arrow) in the coadhesion process. Bar, 500 nm.

Although inhibitory proteins like BSA might interact stereochemically with enzymes or other protein-seeking adhesions on *P. gingivalis*, defatted BSA is known to be a potent inhibitor of microbial adhesion to hydrophobic surfaces (21, 22). It strongly inhibited coadhesion at relatively low concentrations, in contrast to the lower inhibition observed with nondefatted BSA. Such apparent contradictions in the role of hydrophobicity in oral adhesion and coaggregation have been reported previously. McIntire and coworkers found that lactose and sodium dodecyl sulfate (SDS) acted synergistically to inhibit coaggregation between *A. viscosus* T14V and *S. sanguis* 34 (18). SDS itself was 15 times more potent an inhibitor than lactose, whereas neither Tween 80 nor Triton X-100 were inhibitory. Jenkinson and Carter (14) isolated two mutants of *S. sanguis*, with increased and decreased hydrophobicity, compared with the parent strain. The mutant with increased adhesion to hexadecane and octyl-Sepharose coaggregated to a greater extent with *A. viscosus* than the wild type did. However, no differences in coaggregation were observed between the wild type and the less hydrophobic mutant. Clark et al. demonstrated positive correlations between adsorption of oral *Actinomyces* spp. to saliva-coated hydroxyapatite and to hydrophobic gels; however, whereas adsorption to the hydrophobic gel was inhibited by Tween 80, adhesion to saliva-coated hydroxyapatite was unaffected (3). Indeed, the common use of BSA as a blocking agent in oral adhesion studies by researchers interested in isolating the lectinlike or other complementary binding components (7, 27, 30) may underlie their tacit acknowledgment that in its absence, hydrophobic interactions are significant. Concomitant participation of hydropho-

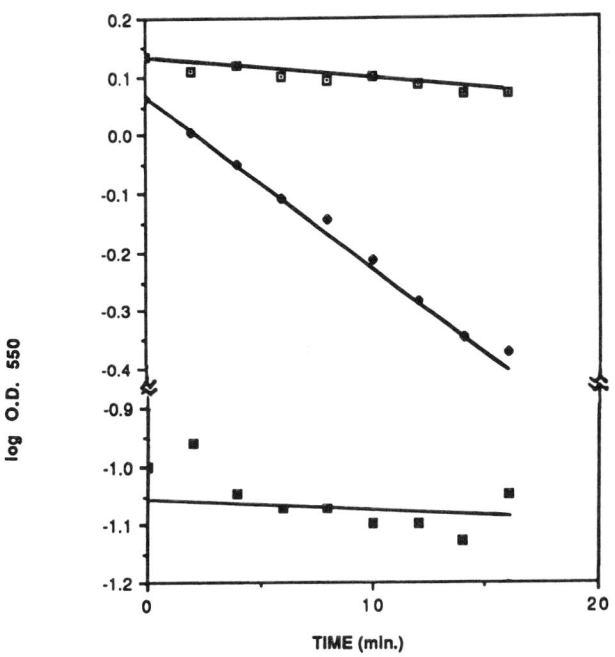

FIG. 7. Kinetics of coadhesion. Results are expressed as the log optical density at 550 nm as a function of time of mixing. Symbols: ◆, *A. viscosus* WVU627 incubated with PCHD; ⊡, control WVU627 cells incubated with hexadecane instead of PCHD; ■, PCHD alone in the presence of buffer.

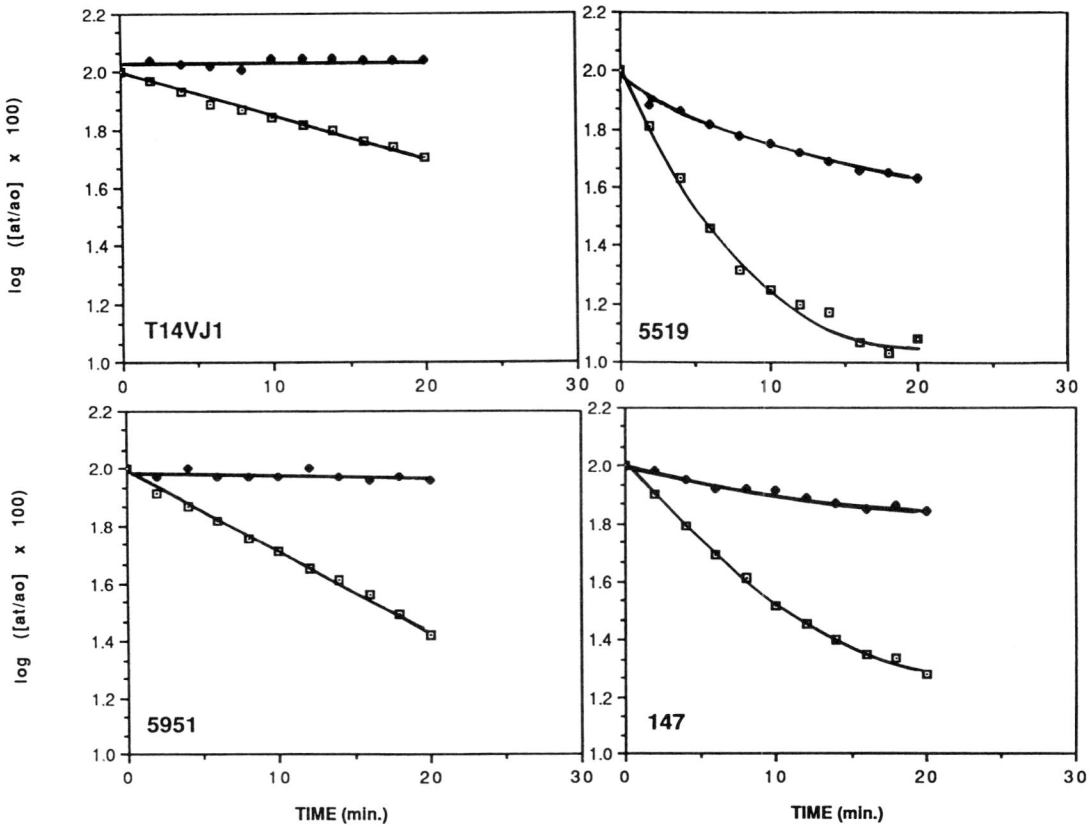

FIG. 8. Coadhesion of *A. viscosus* T14VJ1 and corresponding mutants with PCHD. Coadhesion of *A. viscosus* T14VJ1 and mutant strains 5519 (missing type 2 fimbriae), 5951 (missing type 1 fimbriae), and 147 (missing both fimbrial types) to PCHD was performed as described in Materials and Methods. In each case, *A. viscosus* cells were incubated with PCHD (⊡), as opposed to control tubes (◆), which contained hexadecane instead of PCHD. Results are expressed as the log percent decrease in turbidity [log $(a_t/a_0) \times 100$] as a function of mixing time, where a_t is the absorbance at time t and a_0 is the absorbance at time zero, just before mixing.

bic interactions and stereochemical binding in adhesion of oral streptococci to saliva-coated hydroxyapatite was first proposed by Doyle and coworkers in 1982 (4). However, the mechanism by which this might occur remains unclear.

In coaggregation and coadhesion of *A. viscosus* to *P. gingivalis*, the following model may be proposed. Initial attachment of individual *A. viscosus* cells to *P. gingivalis* cells or cell layers may occur primarily through complementary but not lectin-mediated (1, 7) stereochemical associations (the nature of which is presently under investigation); thus, the preferred adhesion of *A. viscosus* is to *P. gingivalis* rather than to bare hexadecane. However, subsequent attachment of additional *A. viscosus* cells would be greatly promoted by lateral cell-cell interactions with other, bound *A. viscosus* cells, in addition to attachment to the *P. gingivalis* cell layer. Such "like-like" associations may depend more on hydrophobic interactions. This model can account for the increase in BSA-mediated inhibition of coadhesion with time. Furthermore, the tendency of *A. viscosus* cell suspensions to autoaggregate in slide aggregation tests can also be blocked by BSA (data not shown). These data, taken together, strongly suggest that in coaggregation as well as coadhesion studies, possible interactions between cells of the same type should also be considered. The assay system presented here may provide an opportunity for further insight in this direction.

TABLE 1. Comparison of relative hydrophobicity and coadhesion rate[a]

A. viscosus strain	Fimbrial type	% Adhesion to hexadecane	Coadhesion rate (10^{-2} min^{-1})
WVU627	1^+ 2^+	76	2.25
T14VJ1	1^+ 2^+	97	1.44
5519	1^+ 2^-	95	ND[b]
5951	1^- 2^+	89	2.74
147	1^- 2^-	84	ND

[a] Relative hydrophobicity was determined as percent adhesion to hexadecane following 4 min of vortexing (Fig. 1B), and coadhesion rates were derived from the slopes presented in Fig. 7 and 8.

[b] ND, Coadhesion rate could not be determined because of autoaggregation of cells.

The technique described here has several inherent advantages over other assays: (i) multiple readings over time can be made directly in the individual test tubes, (ii) coadhesion can be observed by both light and electron microscopy, (iii) radiolabeling of cells is not required, (iv) no adhesive agents are required to mediate adsorption of the first species to the underlying surface, and (v) as opposed to basic coaggregation techniques, this method depends on rising, rather than sedimentation of, coadhering cells. Thus, the contribution of potential artifacts such as autoaggregation can readily be noted. One basic requirement of the proposed technique is that at least one of the species must adhere to liquid hydrocarbon, and we have demonstrated this limitation with weakly hydrophobic *P. gingivalis* W50. However, since

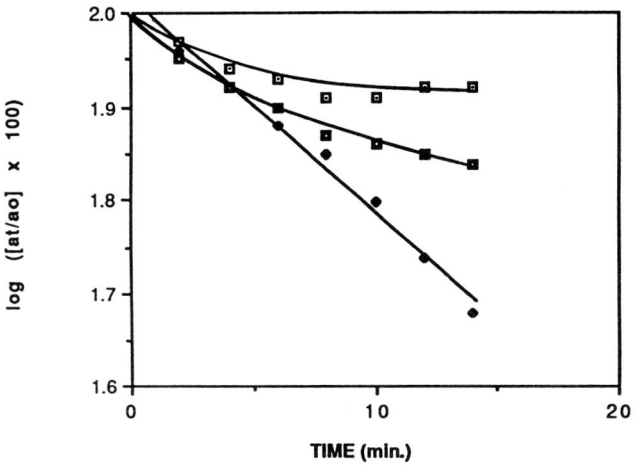

FIG. 9. Effect of BSA and defatted BSA on coadhesion. The kinetics of coadhesion of *A. viscosus* WVU627 to PCHD was measured as described in Materials and Methods in the presence of BSA (■) or defatted BSA (⊡), at final concentrations of 0.2 mg/ml, as opposed to coadhesion in the presence of buffer alone (◆). Results are expressed as the log percent decrease in turbidity [log $(a_t/a_0) \times 100$] as a function of mixing time.

many oral (29) and nonoral (26) microbial strains tested to date share affinity for the hydrocarbon-water interface, this approach may prove a more general one, applicable in studying mechanisms of biofilm coadhesion for bacteria from diverse ecosystems.

ACKNOWLEDGMENTS

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Reproducibility and Sensitivity of Oral Malodor Measurements with a Portable Sulphide Monitor

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Forty-one subjects with bad breath were assessed for oral malodor and periodontal status on three occasions, at intervals of approximately one week. Oral malodor was assessed by measurement of peak and steady-state volatile sulphide levels with a portable sulphide monitor and by organoleptic measurement of whole-mouth, tongue dorsum, and interproximal dental odors by two independent judges. Reproducibility of measurements, assessed by paired *t* tests and Kappa testing, demonstrated no significant differences between any of the test results from the first and second appointments. Steady-state sulphide levels were the most reproducible of all tests. The ability of the tests to detect an expected reduction of malodor following a 0.2% chlorhexidine mouthrinse regimen was investigated by comparison of test values between the second and third appointments. Following the mouthrinsing treatment, 43% reductions of peak, 47% reductions of steady-state volatile sulphide levels, and 15-58% reductions in all other measurement categories were observed. The majority of the participants (22/41) had no pockets greater than 5 mm and exhibited both moderate gingival inflammation (Mean Gingival Index = 1.17) and moderate plaque accumulation (Mean Plaque Index = 1.84). Plaque Index and presence of pockets greater than 7 mm were weakly related to sulphide measurements. Whereas assessment of steady-state sulphide levels by the sulphide monitor does not constitute a direct measure of oral malodor, its relation to organoleptic measurement, superior reproducibility, objectivity, and sensitivity support the use of the sulphide monitor in clinical studies.

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Introduction.

Bad breath (halitosis, oral malodor, *fetor ex ore*) is a common human condition, affecting primarily the adult population (Tonzetich, 1977). Most cases of bad breath originate from microbial putrefaction within the oral cavity itself, and volatile sulphide compounds have been cited as the predominant source of foul odor (Tonzetich, 1978; Kleinberg and Westbay, 1990). In addition to the social aspects of oral malodor, measurement of bad breath may provide a useful screening test for detection of periodontal diseases (Tonzetich, 1978) on the basis of the capacity of subgingival microbiota to produce hydrogen sulphide (Persson *et al.*, 1989).

For the assessment of oral malodor in scientific investigations, reliable methods of measurement are required. Previous published methods of assessing oral malodor have been based upon subjective, organoleptic (hedonic) scales (Nara, 1977; Tsunoda *et al.*, 1981; Ishikawa *et al.*, 1984), or quantitative measurement of volatile sulphide compounds (VSC; Kostelc *et al.*, 1980, 1984), or both organoleptic and VSC measurements (Schmidt *et al.*, 1978). Quantitative measurements by gas chromatography (Solis-Gaffar *et al.*, 1975; Tonzetich, 1977; Kostelc *et al.*, 1984) cryo-osmoscopy (Brening *et al.*, 1939), and culture of plaque and periodontal pocket exudates in bacteriological media (Tachibana, 1957) require elaborate and sophisticated laboratory facilities, and may be time-consuming and expensive. Limited data are available on the reproducibility of such measurements, which may be confounded by circadian variation, menstrual cycle, smoking habits, variations in oral hygiene, climate, and other variables. Moreover, once the gases in the oral cavity are evacuated for measurement, the kinetics of re-establishment to previous levels or proportions is unknown.

Recently, a simple and rapid technique for measurement of volatile sulphide concentration by use of a portable instrument has been proposed (Rosenberg *et al.*, 1991). The aim of the present report was to compare the reproducibility and sensitivity of organoleptic methods for oral malodor assessment with those of the sulphide monitor.

Materials and methods.

Patient population.—Subjects were chosen from among approximately 200 people who responded to an article on bad breath that appeared in a local newspaper. Letters explaining the project were distributed to all respondents, and the first 42 subjects who agreed to participate were enrolled. All subjects reported subjective complaints of bad breath. Two subjects who took antibiotics within two weeks prior to their appointments were deferred for two months prior to starting. There were no exclusion criteria. The total duration of the study was 12 weeks.

Subject preparation.—Subjects were asked to refrain from oral activities, including eating, drinking, chewing, sucking, and mouthrinsing, for at least two hours prior to each appointment. For assessment of reproducibility, measurements were made at the first and second appointments, which were held at intervals of approximately one week. For assessment of the sensitivity of the measurement methods in detecting expected changes in bad breath, patients were instructed to rinse twice daily with a 0.2% chlorhexidine (CHX) gluconate mouthrinse (Contrex Super, Microbex, Markham, ON, Canada). Patients rinsed according to manufacturer's instructions, immediately before bedtime and in the early morning. Each treatment consisted of two consecutive 30-second rinses, with no eating or drinking for at least 30 min afterward. Rinsing was performed over a one-week period from the evening following the second appointment to the evening preceding the third and final appointment.

Volatile sulphide concentration.—VSC measurements were made with a portable industrial sulphide monitor (model 1170, 0.5 ppm, full-scale, Interscan Corp., Chatsworth, CA), zeroed on ambient air before each measurement. A disposable 6.5-mm plastic straw was attached to the air inlet of the monitor. Each patient was instructed to bring his slightly-opened mouth over the straw so that it extended approximately 4 cm into the oral cavity, and to breathe through his nose during the measurement. This instrument contained a pump which sucked air from the plastic straw placed inside the patient's mouth at 1500 mL/min. The sample of mouth air passed through a sensor, which registered the level of sulphides. Both peak (maxi-

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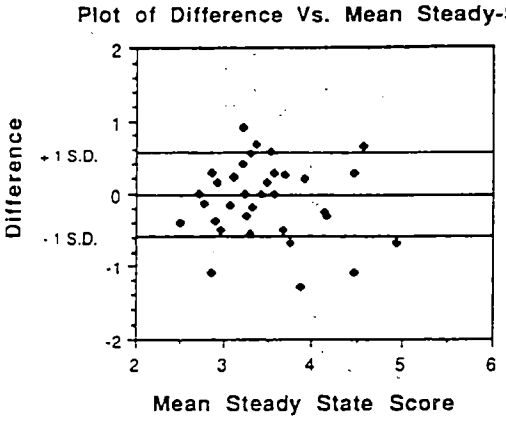
mum) and steady-state levels attained were determined in parts *per* billion (ppb) sulphide equivalents by direct readings from the analog scale of the monitor.

Organoleptic assessment.—Organoleptic assessment was made by two judges, as previously described (Tonzetich and Ng, 1976; Rosenberg *et al.*, 1991). One of the judges had previous experience in measuring several hundred subjects (M.R.). The second judge (A.B.) was an experienced dental hygienist who had not participated previously in organoleptic assessment of malodor. The judges were not pre-calibrated. Organoleptic malodor scores were recorded independently by each judge on a scale of 0 to 5 as follows: 0 - no appreciable odor; 1 - barely noticeable odor; 2 - slight, but clearly noticeable odor; 3 - moderate odor; 4 - strong odor; and 5 - extremely foul odor. Judges were blinded regarding one another's scores, their own previous scores, and VSC measurements. Patients were instructed not to speak for several minutes prior to bad breath measurement.

Tongue malodor was assessed by one of the judges (A.B.) by scraping the dorsum of each patient's tongue with a gauze pad and immediately smelling its odor. Floss malodor was assessed by the passing of waxed dental floss interproximally between all posterior teeth and assessment of the odor following each interproximal passage, based on the above organoleptic scale. Floss was similarly passed between all anterior teeth, but the odor was assessed after passage between all maxillary and mandibular anterior teeth. Different segments of floss were used for each interproximal passage. The highest level of malodor, based on the above organoleptic scale, was recorded.

Measurements of Plaque Index (PI; Silness and Löe, 1964) and Gingival Index (Löe and Silness, 1963) were performed on all teeth at each appointment following malodor measurements. The depth of periodontal pockets was measured with a Michigan O probe (HuFriedy, Chicago, IL) to the nearest mm marking. Unstimulated saliva was collected and scored as present if it exceeded 0.2 mL over a two-minute period.

Statistical analysis.—The reproducibility of the VSC data was assessed by means of a paired *t* test and by plots of differences against means. Intra- and inter-examiner consistencies were assessed by Cohen's Unweighted Kappa Statistic (Cohen, 1960). The sensitivity of the tests in detecting an expected reduction in test values after CHX was assessed by the paired *t* test. The Pearson correlation was used to assess the strength of association between various malodor measurements and periodontal parameters. Multiple regression analysis with the GLM procedure of SAS (SAS 6.03, Cary, NC) was used to examine the relation among steady-state VSC measurements, organoleptic scores, and other patient variables. All analyses were performed with SAS software (SAS 6.03, Cary, NC).



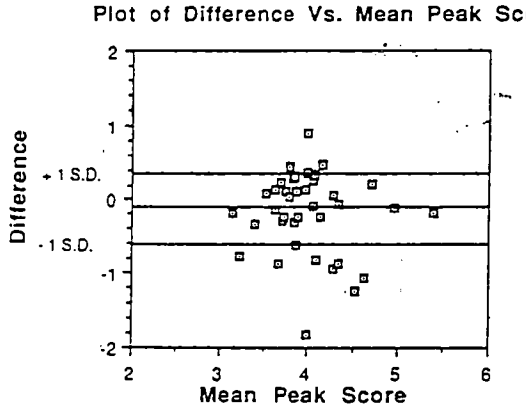


Fig.—Plot of differences between VSC scores at first and second appointments against mean scores for steady-state and peak measurements. Horizontal lines represent one standard deviation from mean. Note that the mean difference is slightly less than zero, indicating slight instrument drift or systematic reduction in VSC between first and second appointments.

Results.

Forty-one subjects participated in this study, of whom 14 were male (47.0 ± 15.3 years; $\bar{X} \pm$ S.D.) and 28 were female (52.3 ± 12.2 years). One person failed to complete the study.

Transformation of peak and steady-state sulphide measurements to natural logarithms (LnPs and LnSs, respectively) yielded near-normal distributions which were checked by means of the univariate procedure and the Shapiro-Wilk test in SAS (Shapiro and Wilk, 1965). Since the possibility existed that the sensitivity of the sulphide sensor decreased with time over the 12-week duration of the study, the decline in the peak and steady-state measurements was estimated between the first and second appointments by computation of the systematic reduction in mean values. Mean peak measurements declined 1.04 ppb/week and mean steady-state measurements by 1.03 ppb/week. Whereas both measurements showed a slight decline, only peak measurements were seen to decline significantly at the 5% level. The finding of declining values with time indicated the possibility of declining instrumental sensitivity. Consequently, all measurements were adjusted for these systematic reductions in sulphide measurement.

No significant differences were found in the adjusted peak and steady-state sulphide measurements made at the first and second appointments (Table 1), and this was true for both sexes (paired *t* test; $p > 0.05$). Plots of differences between peak and steady-state measurements at the first two appointments against their respective means demonstrated that, while all of the data points were within two standard deviations of the overall mean difference, 58% of steady-state values and 48% of peak values were within one standard deviation of the mean, demonstrating the superior reproducibility of steady-state values (Fig.).

Intra- and inter-examiner consistencies of whole-mouth organoleptic measurements were assessed by Cohen's Unweighted Kappa statistic, which assesses the frequency with which examiners can exactly replicate either their own scores or the scores of another examiner. The Kappa statistic provides a rigorous test of examiner agreement, and it is suggested (Landis and Koch, 1977) that a score greater than 0.4 indicates moderate agreement. Intra-examiner consistency ($k = 0.38$ for M.R.; $k = 0.32$ for A.B.) was low but was slightly better than inter-examiner consistency ($k = 0.22$). Consistency in the organoleptic tongue and floss scores between the first and second appointments was similarly analyzed and yielded Kappa values of 0.13 and 0.05, respectively. In contrast to Kappa testing, Pearson correlation analysis indicated a reasonably strong associa-

TABLE 1

MALODOR MEASUREMENTS AND DENTAL PARAMETERS

Measurement	Appointment 1	Appointment 2	Appointment 3
Peak VSC	67.2 ± 7.3	56.2 ± 5.3	35.1 ± 3.0
Steady-state VSC	40.7 ± 5.9	35.6 ± 4.1	20.2 ± 3.1
MR-whole mouth	2.5 ± 0.2	2.5 ± 0.2	1.2 ± 0.2
AB-whole mouth	2.4 ± 0.2	2.3 ± 0.2	1.0 ± 0.2
Tongue score	2.5 ± 0.1	2.5 ± 0.1	1.8 ± 0.1
IP score	3.1 ± 0.2	3.1 ± 0.2	2.4 ± 0.2
Plaque Index	1.8 ± 0.1	1.8 ± 0.1	1.4 ± 0.1
Gingival Index	1.2 ± 0.1	1.1 ± 0.1	0.9 ± 0.1
Saliva	27/11	28/11	29/11
Present/Absent	(missing obs. = 3)	(missing obs. = 2)	(missing obs. = 1)

Values represent mean ± SEM.

tion between the two organoleptic examiners ($r = 0.71$; Table 2) and between the organoleptic assessments and steady-state VSC measurements ($r = 0.60$ for M.R.; $r = 0.52$ for A.B.).

Significant reductions of peak (43%; $p = 0.0001$) and steady-state (47%; $p = 0.0001$) sulphide measurements were observed at the third appointment following the CHX mouthrinse treatment. Similarly, whereas there were no significant differences in the organoleptic odor scores from whole-mouth, tongue, and floss between first and second appointments, they were significantly reduced following the CHX mouthrinse. There were 52% and 58% reductions in the organoleptic whole-mouth ratings, as scored by examiners M.R. and A.B., respectively ($p = 0.0001$). Smaller reductions were observed in the assessment of tongue odor (27%; $p = 0.0001$) and floss odor (22%; $p < 0.005$) scores.

Whole-mouth, tongue, and interproximal (floss) odor scores were found to be independent of the presence or absence of saliva (Chi sq.; $p > 0.05$). Similarly, the peak and steady-state VSC scores, gender, and appointment number were independent of the presence or absence of saliva (Chi sq.; $p > 0.05$). Steady-state and peak sulphide measurements as well as organoleptic scores of malodor were positively related (slope = 0.008; $p = 0.09$) to the age of the subject. This age-related increase was similar in the two sexes and was significantly different from zero ($p = 0.09$); however, there was a large scatter of the data ($r = 0.14$).

Multiple regression analysis of log-transformed steady-state and peak VSC measurements and of organoleptic scores demonstrated that whole-mouth organoleptic scores of one examiner (M.R.), PI, and duration in the study period accounted for most of the variation in the VSC measurements. Individual factors—such as age, gender, GI, organoleptic tongue and organoleptic floss scores—were found to account for a smaller portion of the variability in steady-state scores. These weak associations were also demonstrated by the low r values of individual parameters obtained by Pearson correlation analysis (Table 2). However, when taken together, their overall association with VSC measurements was marked when analyzed by multiple regression ($r = 0.67$).

Twenty-two patients had no periodontal pockets deeper than 5 mm, and the other 19 patients had between one and nine pockets deeper than 5 mm. Three patients had one or more 7-mm pockets (*i.e.*, moderate to advanced disease). Therefore, the majority of these patients exhibited gingivitis without periodontitis (22/41). The mean PI for the first two appointments was not significantly different ($p > 0.05$). At the third appointment following CHX, there was a significant reduction in the mean PI score (26%; $p < 0.0001$).

Patients with one or more pockets greater than 5 mm had steady-state VSC scores 30% higher than those of patients without pockets ($p < 0.05$). A single individual with nine pockets > 5 mm was observed to have steady-state and peak scores higher than those of individuals with fewer pockets (data not shown). However, Pearson correlation analysis demonstrated weak associations between the presence of pockets greater than 5 mm and both organoleptic and VSC measurements (Table 2). No strong formal inferences can be drawn about the relationship between number of pockets and malodor scores, because very few subjects demonstrated severe pocketing, and those with pockets exhibited wide variations in the numbers of pockets.

Discussion.

The sulphide monitor detects primarily hydrogen sulphide. Other VSC's, such as dimethyl sulphide and methyl mercaptan, are also detected, but the detection sensitivity of the instrument to these gases is about 50% of that for hydrogen sulphide (data on file, Interscan Corp., Chatsworth, CA). Since VSC's are known to be important contributors to oral malodor (Tonzetich, 1977), measurement of VSC's made with the monitor could provide a simple and objective approach to malodor assessment. In this report, intra-oral sulphide levels made one week apart in individuals with a chief complaint of bad breath were reproducible, thereby demonstrating an essential prerequisite for the rational use of a diagnostic instrument (Sackett *et al.*, 1985). The reproducibility between the first two appointments was surprising, since we anticipated that confounding factors, such as greater attention to oral hygiene, might have resulted in considerably reduced VSC scores at the second appointment. The instrument was also sensitive to reduced VSC levels resulting from CHX treatment. This expected reduction of VSC levels was consistent with previous data after mouthrinse treatment (Schmidt and Tarbet, 1978) and indicates that the sulphide monitor was sufficiently sensitive for detection of reduced levels of sulphide-containing compounds after anti-microbial treatment.

Previous studies have reported significant correlations between peak sulphide measurements with the sulphide monitor and mal-

TABLE 2

PEARSON CORRELATION ANALYSIS OF MALODOR MEASUREMENTS AND DENTAL PARAMETERS

	LnPk	LnSs	MR	AB	Tongue	Floss	PI	GI	P > 5
LnPk	1.00	0.879	0.552	0.425	0.377	0.208	0.373	0.087	0.280
	0.0	0.0001	0.0001	0.0001	0.0001	0.026	0.0001	0.053	0.002
LnSs		1.00	0.599	0.518	0.353	0.250	0.396	0.067	0.257
		0.0	0.001	0.0001	0.0001	0.007	0.0001	0.479	0.006
MR			1.00	0.714	0.408	0.381	0.353	0.099	0.184
			0.0	0.0001	0.0001	0.0001	0.0001	0.292	0.047
AB				1.00	0.406	0.422	0.359	0.081	0.107
				0.0	0.0001	0.0001	0.0001	0.383	0.250
Tongue					1.00	0.237	0.384	0.255	0.091
					0.0	0.01	0.0001	0.006	0.332
Floss						1.00	0.293	0.189	0.224
						0.0	0.001	0.042	0.016
PI							1.00	0.223	0.006
							0.0	0.016	0.945
GI								1.00	0.143
								0.0	0.124

Pearson Correlation Coefficients/Prob> |R| under Ho: Rho = 0.

Data from all three appointments were analyzed together. LnPk - natural logarithm of peak VSC measurement, LnSs - natural logarithm of steady-state VSC measurements, MR - organoleptic examiner, AB - organoleptic examiner, Tongue - organoleptic score of tongue scraping by AB, Floss - organoleptic assessment of floss by AB, PI - Plaque Index, GI - Gingival Index, P > 5 - number of pockets greater than 5 mm.

odor as determined by a seven-member organoleptic panel (Rosenberg *et al.*, 1991). These reported correlations are consistent with previous data describing gas chromatographic measurements of peak VSC and organoleptic assessments of oral malodor (Schmidt *et al.*, 1978). Since peak measurements may depend on the manner in which the sampling straw is introduced into the oral cavity, and may be greatly influenced by the kinds of oral activities carried out prior to testing (*e.g.*, talking, swallowing, mouth breathing, etc.), steady-state values were recorded as well. Indeed, steady-state values (which indicate the on-going rate of sulphide generation within the mouth) were more reproducible and sensitive to CHX treatment than were peak measurements. Mean steady-state values also increased with age, consistent with early data (Sulser *et al.*, 1939).

The measuring instrument used in this study exhibited an apparent slight downward drift with time. This reduction may reflect a systematic decline in machine sensitivity due to contamination of the H_2S sensor with sulphide-containing components. Consequently, the instrument must be re-calibrated periodically to adjust for the loss of sensitivity. The reduction in scores between first and second appointments may reflect real declines in VSC, possibly induced by improved patient oral hygiene during the study. Verification of the source of this decline awaits the use of highly calibrated standards.

In addition to whole-mouth assessment, we also measured malodor from two intra-oral sites considered to be major foci of malodor, *i.e.*, the dorsum of the tongue and the interproximal areas (Spouge, 1964; Tonzetich and Ng, 1976; Tonzetich, 1977). Whereas bad breath was associated with tongue malodor, interproximal odor, or both in specific patients, neither organoleptic parameter was reproducible, and did not correlate well with the other scoring techniques. Thus, the organoleptic rating of material scraped from these sites by a single observer (A.B.) did not appear to make a significant contribution to overall bad breath measurement in patients with a chief complaint of halitosis. This finding is in contrast to the data of Tonzetich and Ng (1976), who found in normal subjects that dorsal tongue surfaces were the source of the most offensive compounds when assessed by a panel of pre-calibrated judges. It is not clear from the present report whether patients who present with a chief complaint of halitosis may represent a unique population of individuals in whom VSC-producing sites are not restricted to the tongue. Further work is now in progress with site-specific sampling of sulphide compounds from tongue and interproximal sites with a modification to the sulphide monitor.

Based on the superior reproducibility and sensitivity of the sulphide measurements compared with organoleptic measurements, the sulphide monitor may be a valuable tool for assessment of oral malodor. The sulphide monitor also presents other advantages such as: (i) no need for skilled personnel; (ii) non-invasive; (iii) low likelihood of cross-infection; (iv) portability; (v) relatively inexpensive; and (vi) rapid turnaround time of one to two min between measurements. However, it lacks the specificity of gas chromato-

graphy, since it cannot distinguish between the proportions and species of different VSC's. In addition, its utility would be improved if accurate laboratory standards were readily available for daily calibration of the machine, as has been used for calibration of organoleptic measurements (Tonzetich and Ng, 1976).

None of the parameters in the present report can be singled out as a reference standard for the measurement of bad breath. As with other odor measurements, subjectivity is to be expected in any organoleptic assay (Doty *et al.*, 1982). Even in pre-calibrated panels of judges, the calibration step itself may introduce bias (Cain, 1989). The results presented here are consistent with those of many other previous reports (Solis-Gaffar *et al.*, 1975; Tonzetich and Ng, 1976; Schmidt *et al.*, 1978; Kostelc *et al.*, 1984) describing relationships between organoleptic measurements and VSC, and reductions of oral malodor after mouthrinse treatment. The patients examined in this study exhibited appreciable levels of intra-oral volatile sulphides, yet the variance of VSC measurements was not well-explained by variations in salivary flow rate, as has been previously suggested (Tonzetich, 1977). Indeed, in the patient population studied herein, the variance in VSC measurements can best be attributed to the cumulative effect of a number of factors, including the amount of supra-gingival dental plaque, the presence of periodontal pockets, gingival inflammation, and age. If these data can be replicated in an appropriately designed clinical study with adequate sample size and a well-described patient population, then the measurement of oral malodor with the portable industrial sulphide monitor may be useful in the diagnosis of bad breath and the screening of patients for periodontal diseases. Although the strategic application of breath monitoring for periodontal diseases has been previously noted with gas chromatography (Tonzetich, 1978; Kostelc *et al.*, 1981, 1984), the use of a simple device such as the sulphide monitor described here appears to be more applicable to chair-side measurement.

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Halitosis Measurement by an Industrial Sulphide Monitor

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PREVIOUS STUDIES HAVE ESTABLISHED that hydrogen sulphide and mercaptans are the primary components of halitosis (bad breath). In the present investigation, we report a simple, rapid technique for measurement of halitosis-related sulphides. The technique is based on a portable instrument generally used for environmental safety applications. Seventy-five volunteers were measured using this technique, and the results (in peak ppb hydrogen sulphide equivalents) compared with organoleptic assessment by 7 judges. A highly significant overall correlation ($r = 0.603$; $P < 0.001$) was obtained between these 2 methods. Moreover, in most cases, the organoleptic ratings of the individual judges correlated more highly with sulphide monitor values than with one another. The simplicity of the technique suggests its use in clinical studies as well as in diagnosis and treatment of patients with this complaint. *J Periodontol 1991; 62:487–489.*

Key Words: Halitosis, hydrogen sulphide/analysis.

Halitosis is a common condition which affects most of the adult population.[1] In the great majority of cases, halitosis originates in the mouth itself, as the result of microbial putrefaction.[1] Hydrogen sulphide and methyl mercaptan have been cited as the major gases associated with halitosis.[2] Halitosis of oral etiology may result from periodontal diseases, poor saliva flow, improper dental restorations, excessive microbial colonization of the tongue, or unclean dentures.[1,3] Non-oral etiologies include upper and lower respiratory tract conditions, gastrointestinal and neurologic disorders, various systemic diseases, and use of certain drugs.[4] Halitosis is a subject more frequently reviewed than investigated. This is likely due to difficulties associated with its measurement. Recent investigations have relied upon subjective organoleptic (hedonic) ratings by human judges and gas chromatographic techniques.[1,5] Both these approaches are technically difficult and time-consuming. Neither is amenable to quantitative measurement of large subject populations. One possible solution to this problem is the use of portable gas monitors, such as those employed in the environmental health industry. In the present study, we report the applicability of one such industrial sulphide monitor to measure halitosis. Seven organoleptic judges measured the bad breath of 75 volunteers, concomitant with monitor readings. The results suggest that sulphide monitor readings constitute a simple, rapid method for assessing halitosis.

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MATERIALS AND METHODS

The study included 75 volunteers, ranging in age from 11 to 77 years (average age = 47.3 ± 16.7). Among the volunteers, 54 responded to a local radio invitation to participate in the study. These volunteers were tested in the morning, and asked to refrain from oral activities (eating, drinking, brushing, smoking, oral rinsing, etc.) the morning of the testing. The remaining 21 volunteers consisted of dental staff and students who agreed to participate in the study; this group received no prior instructions and was examined concomitantly with the first group. No exclusion criteria were employed.

The organoleptic panel consisted of 3 male and 4 female judges. The judges received prior instruction and details of the scoring system, but did not calibrate or compare ratings. Judges were instructed to refrain from drinking coffee, smoking, or wearing scented personal-care products prior to the examination.

For organoleptic measurements, volunteers were instructed to exhale briefly through the mouth, at a distance of approximately 10 cm from the nose of the organoleptic panel member. Organoleptic results were recorded independently by each judge on a scale of 0 to 5 as follows: 0: no appreciable odor; 1: barely noticeable odor; 2: slight, but clearly noticeable odor; 3: moderate odor; 4: strong odor; and 5: extremely foul odor.

Volatile sulphide measurements were performed using a 1 ppm full scale hydrogen sulphide monitor,[§] with no scrubber (in the absence of scrubbers, the instrument measures

[§]Interscan Corp., Model 1170, Chatsworth, CA.

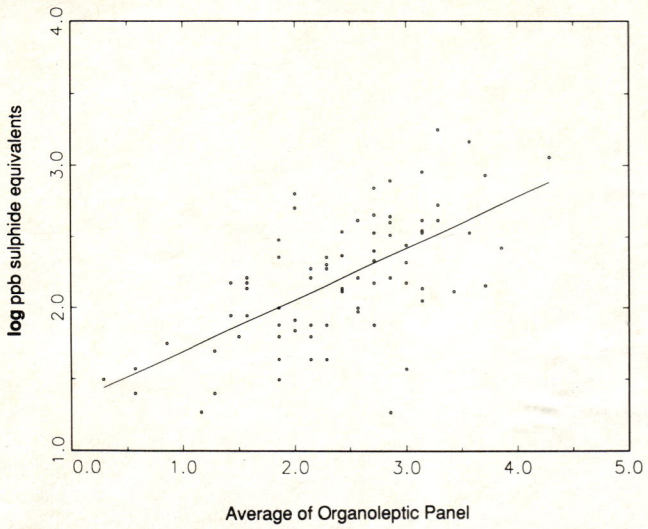

Figure 1. Halitosis measurement by sulphide monitor vs. organoleptic panel. Correlation between sulphide measurements (peak log ppb hydrogen sulphide equivalents) and the average of the judges' ratings (on a scale of 0 to 5) for all 75 volunteers.

both hydrogen sulphide and mercaptans [M. Shaw, personal communication]), connected to a pen recorder. This instrument is based on an enclosed liquid electrochemical cell, through which the gas sample passes at a constant flow rate. The monitor was zeroed on ambient air, and measurement performed by inserting a short length of Teflon tubing (connected to the monitor inlet), fitted with a disposable suction tube approximately 4 cm into the oral cavity. The volunteer was asked to breathe through his/her nose during measurement. Results were recorded as peak ppb sulphide equivalents.

RESULTS AND DISCUSSION

The relationship between the log ppb sulphide concentration and the average of the judges' ratings for all 75 volunteers is shown in Figure 1. As expected, a wide range of scores was obtained. Spearman rank correlation analysis between the log ppb sulphide scores and the average of the judges' ratings yielded a highly significant correlation between the two parameters ($r = 0.603$; $P < 0.001$); the Pearson correlation coefficient was $r = 0.640$; $P = 0.001$. These correlations are comparable to or higher than those previously obtained by Schmidt et al., in a comparison between a trained organoleptic panel and gas chromatographic readings.[5]

The interexaminer correlations are presented in Table 1, alongside the correlations between each judge's ratings and the sulphide monitor results. Interestingly, in 16 out of 20 instances, the organoleptic ratings of the judges correlated more highly with sulphide monitor values than with one another. It is also noteworthy that in several cases, poor correlations were obtained among the judges themselves (e.g., $r = 0.1379$; $P = 0.135$ for judge C vs. judge F). Scores of judges D, F, and G had relatively low correlations both with other judges' scores, and with monitor results. This might attest to their inferior ability as odor judges; nevertheless, the intercorrelation between two of them, i.e., F and G, was highly significant ($r = 0.3570$; $P = 0.001$). Taken together, the data illustrate the large judge-to-judge variations which are possible in organoleptic measurement of bad breath. Whereas the correlations might have been improved by pretraining of judges, such an approach may concomitantly restrict personal judgement.

Several additional factors may play a role in affecting the degree of correlation between the sulphide levels and the scores of the organoleptic panel. The sulphide monitor samples air directly from the oral cavity, whereas the judges

Table 1: Interexaminer Correlations vs. Examiner:Monitor Correlations*

Judge	B	C	D	E	F	G	Monitor
A	r=0.4541 *P<0.001*	0.4719 *<0.001*	0.3338 *0.003*	0.4941 *<0.001*	0.2607 *0.015*	0.4406 *<0.001*	0.4758 *<0.001*
B		0.4228 *<0.001*	0.3202 *0.003*	0.4646 *<0.001*	0.2433 *0.02*	0.4585 *<0.001*	0.4699 *<0.001*
C			0.4283 *<0.001*	0.3776 *<0.001*	0.1379 *0.135*	0.2947 *0.008*	0.4077 *<0.001*
D				0.3970 *<0.001*	0.1849 *0.066*	0.1564 *0.101*	0.3145 *0.004*
E					0.2020 *0.052*	0.3452 *0.002*	0.3767 *0.001*
F						0.3570 *0.001*	0.3205 *0.003*
G							0.3480 *0.001*

*The 7 judges (examiners) are labeled A through G. Spearman correlation coefficients (r) are given for interexaminer ratings, as well as for correlations between individual judges and monitor results (in log ppb sulphide equivalents). The numbers in italics are corresponding probability (*P*) values. The Spearman correlation coefficient between the average of the judges' scores and monitor results was $r = 0.6034$; $P < 0.001$.

rated air which was expelled from the oral cavity, and presumably mixed with lung air. Furthermore, although it has been proposed that hydrogen sulphide and mercaptans are the primary gases causing halitosis,[1,2] organoleptic ratings may be influenced by other gases, degree of humidity, and other factors, which are not measured by the sulphide monitor. Experiments are underway to find ways of further improving the correlations between monitor and judges, and to measure the reproducibility and sensitivity of different modes of halitosis measurement.

In summary, the findings suggest that quantitation of volatile oral sulphides, using a portable sulphide monitor, can provide rapid, objective halitosis-related measurements. Since halitosis of oral origin is usually amenable to standard treatment regimens (e.g., proper oral hygiene and dental treatment, saliva flow stimulation, use of certain mouthrinses), we suggest that longitudinal "chairside" measurements are an essential component of successful diagnosis and treatment of bad breath. Clinical studies are in progress to assess the ability of such measurements to monitor mouthrinse efficacy, changes in periodontal disease activity, and various treatment regimens.

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Basic and Applied Aspects of Microbial Adhesion at the Hydrocarbon: Water Interface

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ABSTRACT: Microbial hydrophobicity has been studied since 1924. During the last decade, various techniques have become available for measuring hydrophobic surface properties of microbial cells. This has led to a surge in investigations suggesting a role for hydrophobicity in adhesion of bacteria to an array of surfaces (oral surfaces, mineral particles, fatty meat, epithelial cells, phagocytes, biomaterials), partitioning at interfaces, as well as gliding mobility. The present manuscript comprises a critical, chronological look at the origins of microbial hydrophobicity research, its development, origins, and applications. Emphasis is placed on microbial adhesion to hydrocarbons, a technique with which the author has the most experience and research interest.

KEY WORDS: adhesion, hydrocarbon, microbial hydrophobicity, interface.

I. INTRODUCTION

Observations related to the partitioning of microorganisms at the oil:water interface date back to the pioneering studies of Mudd and Mudd, published in 1924.[1,2] Interest was revived in the 1970s when hydrocarbons were considered as an inexpensive potential carbon and energy substrate for generating microbial biomass[3] (historical events having since put to rest this line of thought). At about the same time, microbial degradation of petroleum was proposed as a biological approach to pollution control.[3-15] During the past decade, microbial adhesion to hydrocarbon (MATH) has been most frequently studied as a test of cell surface hydrophobicity.[16] The aims of the present manuscript are to (1) present a brief chronological account of the study of microbial partitioning at the oil:water interface, including its development as the MATH assay; (2) evaluate the MATH assay and its significance; and (3) discuss various applications based on adsorption of microorganisms and macromolecules to hydrocarbon droplets. For a more general discussion of the relationship between microbial adhesion to hydrocarbons and cell surface hydrophobicity, the reader is referred to several recent reviews.[17-22]

II. MICROBIAL ADHESION TO HYDROCARBONS: CHRONOLOGY

Mudd and Mudd[1,2] studied the behavior of microorganisms at oil:water interfaces, primarily as a model for furthering understanding of phagocytic processes. Following publication of their observations and theoretical model in back-to-back papers in 1924, they appear to have abandoned interest in microbial partitioning at the oil:water interface. Indeed, the scientific community took little interest in this phenomenon for the next 50 years or so. In an autobiographical review, published in 1969, Stuart Mudd barely referred to these studies ("Emily and I were preoccupied at this time with the movement of particles such as bacteria along interfaces and through capillary spaces...which we hoped would

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throw light on the early stages of bacterial invasion'').[23] Today, these investigations are considered as prescient forerunners of the field of microbial cell surface hydrophobicity. In 1931, Reed and Rice demonstrated quantitatively that non-wettable microorganisms (mycobacteria) are able, upon mixing, to pass from the aqueous into the bulk oil phase.[24] However, their attempts to quantify the observation of Mudd and Mudd of bacterial partitioning at the interface itself were not successful. This was presumably due to their less-than-fortuitous choice of strains. Had they used the strain *Erythrobacillus prodigiosis* (a pigmented strain of *Serratia*) of Mudd and Mudd, this field might not have suffered such a lengthy subsequent hiatus.

In the early 1970s, interest was renewed in microbial adhesion to oil. The studies of Marshall and co-workers on the oriented adhesion of *Flexibacter* and *Hyphomicrobium* at the oil:water interface[25] were followed by a classic book on microbial adhesion[26] which emphasized the potential contribution of the hydrophobic effect. Concomitantly, van Oss and co-workers undertook a reevaluation of the role of cell surface hydrophobicity in phagocytosis, being the first to employ contact angle measurements on bacterial layers.[27-29]

In the meantime, petroleum microbiologists had begun studying the role of adhesion to oil in growth in microbial fermentations[4,5-7,9-13,15] as well as *in situ*. McLee and Davies[8] reported linear growth of *Torulopsis* on hydrocarbon. Kappeli and Fiechter[6] measured the partitioning of radiolabeled hexadecane to the cell surface of *Candida tropicalis*, and showed that detergents (Tween 80 and Triton X-100) reduced adsorption. Neufeld and co-workers[11] observed that during growth of *Acinetobacter calcoaceticus* on hexadecane in a batch fermentor, the entire cell population bound to the oil:water interface. Considerable discussion took place as to whether growth of bacteria and fungi on oil required adhesion, ''pseudosolubilization'' of the insoluble hydrocarbon phase, or a combination of both.

It is interesting to note that adhesion researchers in the area of petroleum microbiology appear to have been oblivious to the earlier work of Mudd and Mudd,[1,2] as well as the contemporary investigations of Marshall and co-workers,[25,26] demonstrating that nondegraders of hydrocarbon such as *Serratia marcescens*, *Hyphomicrobium*, and *Flexibacter*, partitioned at oil:water interfaces. In their 1975 paper, Kennedy et al.[7] studied adhesion to hydrocarbon of microorganisms which did utilize hydrocarbons as sources of carbon and energy vs. *Escherichia coli*, which did not. The latter microorganism did not adhere to hydrocarbon, leading the authors to conclude that the ability to partition at the oil:water interface was a property unique to petroleum-degrading bacteria. This overgeneralization was made in other laboratories as well.[9,30] For example, Miura et al.[9] found that hydrocarbon-degrading strains of *C. intermedia* and *C. tropicalis* were much more adherent to hydrocarbon (n-tetradecane) than *Saccharomyces cerevisiae*, a nondegrader.

In the early 1970s, Eugene Rosenberg, David Gutnick, and co-workers initiated a 15-year joint project in petroleum microbiology.[3] Enrichment culture techniques yielded several interesting strains capable of growing on different petroleum fractions.[5,12] One adherent strain, *A. calcoaceticus* RAG-1, was found to elaborate a potent polyanionic heteropolyaccharide bioemulsifier, emulsan.[31,32] Emulsan appeared to show specificity in its emulsification of various hydrocarbon combinations.[33] It was thus of interest to determine whether emulsan, while still bound (in the form of a minicapsule) to the cell surface, prior to its release, was the cell surface adhesin mediating adhesion of RAG-1 to hydrocarbons.

While trying to develop a simple assay to study microbial adhesion to solid paraffin, we chanced upon a phenomenon which (not appreciating at that time the relevance of the research of Mudd and Mudd) we found quite surprising. *Serratia marcescens* cells, mixed vigorously in the presence of liquid paraffin (n-hexadecane), disappeared completely from the aqueous phase and could be observed bound to the surface of the hydrocarbon droplets. Thus, just by vortexing washed cell suspensions with liquid hydrocarbons, adhesion (or lack thereof) could be observed. It soon became apparent that adhesion to hydrocarbon was not specific for oil degraders, as we had anticipated.[34] Various nondegraders adhered avidly to liquid hydrocarbons (e.g., *Streptococcus pyogenes, Staphylococcus aureus*)

whereas some hydrocarbon degraders (e.g., *Pseudomonas aeruginosa*) adhered poorly. Moreover, there did not appear to be a specificity for adhesion to metabolizable substrate since RAG-1 adhered to both hydrocarbons which it could (*n*-hexadecane) or could not (*n*-octane, *p*-xylene) degrade with comparable affinity. It appeared, however, that the test distinguished so-called "hydrophobic" bacteria (*A. calcoaceticus, S. marcescens, S. aureus*) from "nonhydrophobic" ones. (*S. epidermidis, E. coli*, exponentially growing *S. marcescens*). Furthermore, the cells could be desorbed by surface-active agents, such as isopropanol. Thus, after some deliberation, bacterial adhesion to hydrocarbons (BATH), later renamed MATH[19] (microbial adhesion to hydrocarbons), was proposed as a simple assay for measuring "microbial cell-surface hydrophobicity."[16] Interestingly, Iimura and co-workers[35] independently proposed a similar type of assay, but employing much higher hydrocarbon:water volume ratios, and used it to compare different fungal strains.

III. THE MATH ASSAY

The MATH assay has been most frequently used as a test for measuring "cell-surface hydrophobicity". Other commonly used techniques include contact-angle measurement (CAM),[27-29] hydrophobic interaction chromatography (HIC),[36] and the salt aggregation test (SAT).[37] The degree of agreement between these assays is a matter of some debate, and has been recently reviewed.[19,22] In many, but not all, instances, results obtained using MATH correlate with other so-called hydrophobicity tests [19,22] (this subject is discussed further in Section IV).

According to the MATH assay as originally proposed, turbid washed suspensions of microbial cells (1.2 ml) are vortexed in the presence of 0.01 to 0.2 ml of liquid test hydrocarbon (*n*-hexadecane, *n*-octane, *p*-xylene). During the mixing procedure, the liquid hydrocarbon layer is dispersed into droplets which may adsorb microbial cells. In the case of nonadherent cells, the droplets quickly coalesce, and the turbidity in the lower aqueous phase remains unchanged. When adhesion takes place, however, the cell-coated droplets rise to form a stable "cream", and the turbidity of the lower aqueous phase decreases. The percent of adhering cells can be determined simply by measuring this drop in turbidity.

Over the past decade, several modifications in the basic assay have been adopted by many laboratories using the test. The original PUM buffer is often replaced by P buffer (i.e., 150 m*M* phosphate buffer without the urea and magnesium sulfate).[38] The assay can be performed at different pH values.[39] High salt concentrations might be added, e.g., to test halophilic microorganisms.[40] In the original technique, test tubes were preincubated at 30°C prior to the assay.[16] This step has also been largely abandoned despite its potential importance in improving reproducibility. Clearly, performing the test with *n*-hexadecane, at room temperatures close to its solidification point (16°C), may have a large effect on hydrocarbon viscosity and thus droplet size. In the original assay,[16] the lower aqueous phase was removed with a Pasteur pipette to a separate cuvette for spectrophotometric measurement. However, if care is taken to ensure that the hydrocarbon droplets lie above the light beam, the assay can be performed directly in a cuvette or any test tube which fits into a photometer. Whereas the original assay was based on three test hydrocarbons, the assay is often currently employed using a single hydrocarbon (usually *n*-hexadecane). Vanhaecke and Pijck[41] have shown that hexadecane does not damage the intactness of microbial cells during the assay; similar results were obtained with octane. Xylene, on the other hand, resulted in cases of cell lysis and its use as test hydrocarbon should be avoided, if possible.

The original assay was performed with test tubes which had been acid-washed. Since surfactants interfere with the assay,[38] care must be taken in using clean laboratory ware. It has recently been found[119] that commercially produced *n*-hexadecane (including those stated to be >99% pure) may contain contaminants that interfere with the assay. In our hands, *n*-hexadecane recently purchased from Sigma Ltd. (St. Louis) gave highly reproducible results. Hexadecane can be protected from undesirable oxidation by storage under nitrogen gas. In any case, a simple test can determine whether the assay mixture is relatively

surfactant-free: the buffer and test hydrocarbon are vortexed under the assay conditions. If the droplets do not coalesce rapidly, or if a stable emulsion is formed, an interfering contaminant is likely present.

The main advantage of the MATH assay is that it is so easy to perform and can be carried out with equipment found in virtually every microbiology laboratory. Since it is a rapid test, there is little chance for the microorganisms to undergo cell-surface changes as a result of ongoing metabolism or prolonged incubation. Secondly, the adherent cells rise rather than sediment following the assay, so there is little chance of confusing settling or clumping of cells with adhesion.[19] Similarly, no washing steps (which can result in desorption or translocation of cells, particularly if air:substratum interfaces are generated during the washing procedure[42,43]) are necessary to distinguish adherent and nonadherent cells. Another advantage is the possibility of direct observation of the cells adhering to the substratum in the phase,[16] light,[19,44] or electron[45] microscope. Finally, in contrast to many assays employing solid substrata in which only a small fraction of the cell suspension actually comes in contact with the surface, in the MATH assay, the adhesion characteristics of the suspension as a whole are measured.

An important drawback of the MATH assay is that steady-state conditions are practically unattainable. The more one vortexes the mixture, the smaller the droplets, and the greater the surface available for adhesion. Moreover, cells with high affinity for the hydrocarbon:water interface do not appear to desorb under normal assay conditions. These observations prompted several researchers to propose a kinetic approach[46,47] in which adhesion to increasing hexadecane volumes is followed as a function of time. This modification yields removal rates for each hydrocarbon volume. Plotting of the removal rates as a function of the hydrocarbon:water volume ratio yields a straight line, the slope of which is termed the "removal coefficient". In most instances studied to date, back extrapolation of this line to a hydrocarbon:water volume of zero (i.e., no hydrocarbon) does not yield a zero adhesion rate, as one might expect, but rather a finite positive value. This phenomenon has yet to be adequately explained.[47] Another potential problem is that, at least in the case of *A. calcoaceticus* RAG-1, the kinetics of adhesion are dependent on the initial cell density.[119] Thus, in any comparison among closely related strains, or following various treatments, the initial cell densities of the suspensions must be comparable in order to draw conclusions regarding relative affinities for the test hydrocarbon.

IV. APPLICATIONS

A. Isolation of Mutants with Altered Surface Properties

With certain strains, adhesion levels of over 99% to hexadecane can be attained. Under such conditions, nonadherent mutants may be easily enriched from the lower aqueous phase. Rosenberg and Rosenberg[48] used such a technique to isolate the nonhydrophobic mutant strain (MR-481) of wild-type *A. calcoaceticus* RAG-1. A washed cell suspension was vortexed with *n*-octane. Following phase separation, the lower aqueous phase was removed to a fresh tube containing *n*-octane and the process repeated until no observable turbidity remained in the aqueous phase. The lower phase was then inoculated into growth medium. Following growth to stationary phase, the culture was washed, and the process repeated. Following four such growth and extraction cycles, turbidity remained in the lower aqueous phase even following repeated vortexing with octane. Several nonadherent and semi-adherent clones were obtained. One such clone, MR-481, was devoid of thin fimbriae present on wild-type cells, as well as other (as yet unidentified) antigens.[49]

A similar process was used to isolate mutants of *S. marcescens* RZ, deficient in adhesion properties, except that hexadecane was used rather than octane.[50] In this instance, the colonies which were isolated differed in their visual characteristics, as well as in their ability to adhere to hydrocarbons and polystyrene. One mutant, 3162, was devoid of the lipophilic pigment, prodigiosin. Another was pigmented, but colonies were translucent with respect to those of wild-type cells. Comparison of the wild-type and mutant strains

thus suggests that the hydrophobic pigment, prodigiosin,[50] and a cell-surface protein (serraphobin[51]) contribute to the adhesion properties of wild-type cells.

Three laboratories have employed this approach to study the surface properties of *Streptococcus sanguis* strains. Gibbons and coworkers[52] isolated a nonhydrophobic mutant of *S. sanguis* FC-1 and found that it was deficient in adhering to saliva-coated hydroxylapatite (sHA), as compared to wild-type cells. Mutant cells were devoid of fimbriae present on wild-type cells. Morris and co-workers[53] isolated nonadherent mutants of strains 12 and found them deficient in adhesion to sHA and in saliva-mediated aggregation. Mutant cells were deficient in a 160-kDa mol w surface protein implicated in aggregation, were denuded of surface fibrils present on wild-type cells, and secreted a number of smaller proteins not observed in wild-type supernatants. More recently, Jenkinson and Carter[54] found mutants of strain OB11 (Challis) which were either less adherent, or more adherent, to hexadecane than wild-type cells. The second class of mutant may have become attached to the thin film of hexadecane on the glass pipettes used to transfer the aqueous phase. These authors showed a correlation between adhesion to hexadecane, adhesion to sHA, and coaggregation between *S. sanguis* and *Actinomyces viscosus* cells. Two surface proteins, of 43 and 45 kDa mol w, appeared to be associated with adhesion to hexadecane.

Wolkin and Pate used this method to select for nonadherent mutants of *Cytophaga johnsonae*.[55] The seven mutants isolated in this manner were invariably deficient in their spreading abilities. Interestingly, they concomitantly gained phage resistance and lost the ability to degrade chitin. Selection for mutants of *Flexibacter* FS-1 nonadherent to hexadecane similarly yielded strains deficient in spreading ability.

More recently, Yamada and Matsumoto employed this technique to isolate mutants of *S. aureus*.[56] The investigators found that adhesion to octane correlated with elicitation of chemiluminescence response of human phagocytes. Mutants of protein A-positive wild-type strains were not devoid of this surface protein, suggesting that protein A is not responsible for adhesion to hydrophobic surfaces in the strains tested.

These studies mentioned underline both the advantages and disadvantages of employing nonadherent mutants to study adhesion and related properties of wild-type cells. Certainly, comparison of the adhesion of wild-type vs. mutant cells to hydrocarbons and various other surfaces (e.g., teeth,[57] buccal epithelial cells,[58] catheters[59]) has helped to demonstrate that adhesion to hydrocarbon appears closely related to adhesion to other substrata of interest.[19,38] On the other hand, cell surface mutants may often lose several components (or functions) simultaneously, making it hard to pinpoint the factor(s) actually involved in the adhesion process. Moreover, the components mediating adhesion to hydrocarbons and other surfaces may vary among different strains of the same, or related, species.

B. Microbial Treatment of Oil Pollution

In recent years, both accidental and intentional release of crude oil into open waters has created potentially catastrophic environmental consequences. One approach to treating such oil spills is by seeding them with hydrocarbon-degrading microorganisms. In 1981, Rosenberg and Rosenberg demonstrated that, in a poorly mixed system, adhesion is crucial for growth on hydrocarbon.[48] Growth of adherent *A. calcoaceticus* RAG-1 cells in test tubes overlaid with hexadecane, and incubated with limited agitation, proceeded almost immediately following inoculation. However, nonadherent mutant MR-481, isolated as described in the previous section, was unable to grow under these conditions, unless exogenous emulsifier was added to disperse the hydrocarbon substrate. It was subsequently demonstrated that prolonged incubation of MR-481 in the absence of emulsifier enriched for revertants possessing partial adhesion properties.[49] Thus, in a closed system with oil which has been dispersed by emulsification, growth can take place without adhesion of cells. However, in an open system, microorganisms and dispersed hydrocarbon droplets would tend to diffuse away from one another in the absence of direct contact.[60] Since oil spills are extremely slow to disperse, it seems reasonable to assume that adhesion at the oil:water interface may be a desirable char-

acteristic in strains intended for inoculation into such areas. Nevertheless, in discussions regarding microbial degradation of oil spills, the aspect of adhesion is often overlooked.[61] *In situ* studies are necessary to substantiate the apparent advantage of using adherent strains for such purposes.

C. Hydrophobization of Microbial Cells

In general, surfactant molecules tend to inhibit microbial adhesion to hydrocarbons and to other hydrophobic surfaces.[19] However, it has recently been shown that appropriate concentrations of two cationic surfactants, cetylpyridinium chloride and chlorhexidine, can increase microbial adhesion to hexadecane from near 0 to over 90% levels.[62] Microorganisms tested included *E. coli*, nonadherent mutant MR-481, derived from *A. calcoaceticus* RAG-1 as previously described, and a poorly adherent strain of *C. albicans*. Similar enhancement of adhesion to hydrocarbons was subsequently demonstrated for larger, cationic polymers, i.e., chitosan, poly-L-lysine, and lysozyme.[63] Adhesion mediated by these cationic molecules appears to occur by their adsorption to the cell surface, thus diminishing the negative surface charge and increasing the apolar character of the outermost cell surface. Many microorganisms of considerable basic and applied scientific interest, i.e., most *E. coli* strains and vegetative *Bacillus* cells, do not adhere to hydrocarbon. The "hydrophobization" conferred upon them by organic cationic molecules may enable them to adhere avidly to hydrocarbons. This in turn suggests that adhesion to hydrocarbon droplets could constitute a general method for long-term immobilization of microbial cells for various purposes.[63]

D. Study of Mixed Natural Populations

Two aspects of the MATH assay render it suitable for studying population of mixed cells: (1) adhesion is measured turbidimetrically, thus affording a rough estimate of the proportion of adhering cells; and (2) the types of adhering cells can be observed and verified microscopically. This approach has been used to demonstrate adhesion to hydrocarbons of bacteria derived directly from dental plaque,[64] oral expectorate,[62] and human feces.[38].

E. Bacterial Desorption and Mouthwash Development

In 1982, Nesbitt et al. reported the adhesion of *Streptococcus sanguis* to toluene.[66] In the same year, Weiss et al.[65] reported that a high proportion of adherent oral microbial isolates retrieved from extracted teeth and steel bands incubated within the mouth adhered to hexadecane. In 1983, Rosenberg et al. demonstrated that washed, dispersed suspensions of microorganisms obtained directly from supragingival dental plaque adhered in high proportions to test hydrocarbons.[64] These initial observations led to the notion of using two-phase oil:water mouthwashes for *in vivo* desorption of oral microbiota. Indeed, subsequent studies have demonstrated that a wide variety of laboratory strains of oral microorganisms, including *Actinobacillus actinomycetemcomitans, Actinomyces viscosus, A. naeslundii, Bacteroides (Porphyromonas) gingivalis, C. albicans, Streptococcus salivarius,* and certain *S. mutans* strains, adhere with high affinity to hydrocarbons (for a recent review, see Doyle et al.[67]). Initial experiments showed that microorganisms which adhere to hydrocarbons also bind to various nontoxic oils.[68] This suggested that two-phase, oil:water mouthrinses might be of clinical value in desorbing oral microorganisms. *In vitro* experiments showed that aqueous:hexadecane and aqueous:olive oil combinations removed over 97% of a film of *A. calcoaceticus* RAG-1 cells on polystyrene cuvettes.[68] As mentioned earlier, adhesion of oral microorganisms to oil could be further promoted by low concentrations of a cationic antibacterial agent (cetylpyridinium chloride) which is commonly added to mouthwashes. Prototype formulations, consisting of an aqueous phase containing CPC and an oil phase comprising a mixture of vegetable and essential oils, were subsequently developed. Such formulations maintained dramatic *in vitro* bacterial desorption properties,[69] as compared to commercial mouthwashes. In an initial clinical study, significant decreases in oral microbial activity and bad-breath-associated volatile sulfides were noted

some 8 to 10 h following use of an oil:water formulation, as compared to a placebo rinse.[120]

F. Protein Separation and Adhesin Identification

One intriguing aspect of MATH is the effect of phase transition of the liquid hydrocarbon. When hexadecane droplets bearing adherent bacteria are cooled to solidification (below 16°C) and then allowed to melt at room temperature, the droplets coalesce and the bacteria are desorbed into the bulk aqueous phase.[38] The mechanism by which this occurs is not clear. However, it was subsequently found that amphipathic proteins may behave in a similar manner.[70] When a commercial mixture of seven Sigma marker proteins (Sigma Ltd., St. Louis) was vortexed in the presence of hexadecane, one of the proteins (glyceraldehyde 3-phosphate dehydrogenase) disappeared from the aqueous phase. When the hexadecane was solidified and subsequently allowed to melt, the protein reappeared in the lower aqueous phase. Commercial and salivary lysozyme were also extracted from the aqueous phase and then desorbed in active form from the hexadecane:aqueous interface, using this phase transition approach.[70] Adsorption and desorption has similarly been shown for apolipoprotein E and hemoglobin.[121]

Perhaps, most interesting in the present context is the possibility of isolating and identifying adhesins using this procedure. In *S. marcescens* RZ, a major 70-kDa outer surface protein, serraphobin, is produced by hydrophobic wild-type cells, but not by mutants deficient in adhesion and hydrophobic surface properties.[51] Serraphobin adsorbs to hexadecane droplets and can be desorbed and purified by the phase transition technique, providing additional support for its role in mediating adhesion of *S. marcescens* to hydrocarbons and polystyrene.[51] It would be of interest to attempt to purify structural adhesins, e.g., fimbriae, using this simple technique (see also Section IV).

G. Microbial Pathogenesis and Diagnostics

Various microorganisms with pathogenic properties have been shown to adhere in the MATH assay.[16,19,38] In 1983, Ofek, Whitnack, and Beachey proposed that *Streptococcus pyogenes* cells are hydrophobic and adherent during the initial stages of infection (i.e., adhesion and colonization) and subsequently elaborate a hydrophilic hyaluronic acid capsule which protects them from phagocytosis, once they have penetrated the mucosal barriers. In some cases, so-called nonhydrophobic mutants appear to be less pathogenic than wild-type strains. Nevertheless, Rumelt and co-workers were unable to link MATH and colonization of mice by *S. marcescens*.[72,122] Clearly, more work needs to be done to clarify the circumstances under which the ability to adhere to hydrophobic surfaces is related to pathogenesis.

The above notwithstanding, adhesion to hydrocarbons may have considerable diagnostic value. Martin and co-workers[73] have studied MATH, using toluene, as a test for pathogenic coagulase-negative staphylococci. Among the various tests employed (slime production, species determination), adhesion to toluene had the highest positive predictive value (79%) in identifying pathogenic isolates. Using a somewhat similar approach, Boujaafar et al.[74] recently studied adhesion to *p*-xylene of 88 clinical *Acinetobacter baumannii* isolates. Among 65 potentially pathogenic strains, isolated from infected catheters and tracheal and bladder devices, a high proportion (92%) were adherent. In contrast, among 23 isolates obtained from the skin of healthy individuals, only two adhered appreciably to the test hydrocarbon. Thus, at least for some bacterial species, the MATH test may prove

a valuable diagnostic test for distinguishing pathogenic isolates.

H. Spore Adhesion, Separation, and Flotation

Early observations that bacterial spores are more hydrophobic than the vegetative cells from which they originated[75,76] were based on the increased ability of spores to be adsorbed by air bubbles and enriched in the upper foam (foam flotation). More recently, a variety of investigations have shown that bacterial spores adhere to hydrocarbons in much greater proportions than the corresponding vegetative cells.[77-82] Indeed, foam flotation and adhesion to hydrocarbons are much more similar processes than was previously realized.[63]

Flotation may be of potential importance in biological treatment of mosquito-infested waters, using larvicidal spores *(Bacillus thuringiensis israelensis, B. sphaericus)*. Such spores kill mosquito larvae, but since the larvae grow at the water surface, the spores must be prevented from sinking. One possibility is flotation of such spores on the surface of biodegradable, vegetable oil droplets. Although adhesion of larvicidal spores to hexadecane and corn oil was not high in phosphate buffer (14 to 47%), high levels of adhesion were obtained in the presence of 2 *M* ammonium sulfate (85 to 91%).[83] However, it is not known whether these bound spores remain active against the targeted larvae.

Mixtures consisting of vegetative cells and endospores of a given strain can also be vortexed with hydrocarbon. In such instances, the spores will usually partition at the interface, with the vegetative cells remaining in the bulk aqueous phase.

I. Microbial Coadhesion

Certain microbial cells tend to bind to cells of other strains. This phenomenon may be of importance in establishment of complex microbial biofilms, such as dental plaque. Initially, this phenomenon was studied by mixing aqueous suspensions of two strains, and observing the visible "coaggregation".[84] Despite the simplicity of this approach, its ecological relevance is unclear. Thus, investigators have recently begun to study coadhesion of one strain, free in suspension, to a preformed layer of the complementary strain on a given surface. Liljemark and co-workers[85] employed a continuous layer of *Streptococcus sanguis* cells, bound to plastic and enamel surfaces using a commercial adhesive, in order to study coadhesion by *Haemophilus parainfluenzae* and *Streptococcus sobrinus*. Ellen et al. studied attachment of *B. (Porphyromonas) gingivalis* to preformed layers of *A. viscosus* on saliva-coated hydroxyapatite beads.[86] Considerable difficulties are associated with preparing and treating such surfaces and subsequently measuring coadhesion of the second species. One possible alternative is adhesion of one of the species to hydrocarbon droplets, followed by measurement of the coadhesion of the second species. This has recently been demonstrated for coadhesion of free *A. viscosus* cells to hexadecane droplets which were previously coated (by a simple vortexing procedure) with *P. gingivalis*.[45] Since coadhering cells of *A. viscosus* were removed from the bulk aqueous phase to the surface of *P. gingivalis*-coated droplets, the kinetics of coadhesion could be measured directly in individual test tubes. One interesting observation was that, whereas *A. viscosus* cells adhered to hexadecane following vigorous agitation, they did not adhere to bare patches on the hexadecane droplets under the mild mixing conditions of the coadhesion assay.[45]

V. IMPLICATIONS AND FUTURE PROSPECTS

The inherent simplicity of taking a suspension of washed microbial cells, mixing it with hydrocarbons, and observing adhesion has made it a popular assay during the last decade. Some recent investigations which employed this technique are listed in Table 1. Many correlations have been found between the ability of microorganisms to bind to hydrocarbons and to adhere to a wide variety of solid surfaces.[38] Interestingly, such correlations include not only surfaces with acknowledged hydrophobic properties (e.g.,

TABLE 1
Some Recent Studies on Adhesion to Hydrocarbons

Subject	Ref.
Correlation between turbidimetric and ATP assessment of adhesion to hydrocarbon	41
Coadhesion of *Actinomyces viscosus* to *Porphyromonas gingivalis*	45
70-kDa hydrophobin of *Serratia marcescens*	51
Use of MATH to isolate cell-surface mutants	54, 56
Phagocytosis of *Staphylococcus aureus*	56
Enhancement of adhesion by cationic surfactants and biopolymers	62,63
Use of MATH for diagnosis of clinically relevant isolates	73,74
Adhesion and hydrophobicity of spores	79—82
Adhesion of *Pseudomonas aeruginosa* to stainless steel	88
Hydrophobicity measurement of mineral and pigment particles	96
Adhesion vs. emulsification in growth of *Pseudomonas* on hexadecane	97
Effect of 6-aminpenicillanic acid and chloramphenicol on adhesion of *P. aeruginosa* to *m*-xylene	98
Opsonic requirements and adhesion to xylene of *Staphylococcus epidermidis*	103
Adhesion of *Candida albicans* to plastic	104
Adhesion of Streptococcus mutans to saliva-coated hydroxylapatite	105
Adhesion of *P. aeruginosa* to soft contact lenses	106
Bacterial attachment to meat surfaces	107
Effect of various treatments on adhesion of encapsulated and nonencapsulated strains of *Pasteurella multocida*	108
Effect of iron depletion on adhesion and phagocytosis in *Staphylococcus aureus*	109
Effect of growth rate on adhesion of *Streptococcus salivarius* to hexadecane	110
Adhesion of *Straphylococcus epidermidis* and *S. aureus* to intravascular catheters	111
Agglutination of neuraminidase-treated erythrocytes by *Bacteroides intermedius*	112
Effect of potassium sorbate on adhesion of *Alteromonas putrefaciens* to octane	113
Effect of antiserum on adhesion of *Klebsiella* to octane	114
Adhesion of invasive and carrier *Neisseria meningitidis* strains to *p*-xylene and buccal epithelial cells	115
Adhesion and hemagglutination of *Renibacterium salmoninarum*	116
Capsule reduces adhesion to xylene in *Klebsiella pneumoniae*	117
Oxygen transfer using *n*-dodecane as a vector	118

polystyrene and other plastics),[87] but also increasingly wettable surfaces such as stainless steel[88] and even mercury.[123] These data suggest that microbial adhesion to many surfaces may be more dependent on exclusion from the bulk aqueous phase than affinity for the surface itself.[38]

Several recent observations suggest that, whereas MATH is a simple test to perform, it is not a simple test to interpret and understand. For example, why do *A. viscosus* cells adhere to hexadecane only following vortexing and not when mixed under gentle agitation?[45] Is there an energy barrier which must first be overcome to enable adhesion at the oil:water interface? Certainly, once adhesion to hydrocarbons has occurred, it is difficult to reverse. Cell-coated hydrocarbon droplets are usually stable at room temperature for periods of days to weeks.[38,45] Moreover, oil droplets can desorb cells from solid surfaces, thus suggesting that adhesion to oil is energetically favorable over adhesion to solid surfaces. One possible explanation for this is that the oil surface deforms around the adherent microorganism,[2] thus maximizing the area of contact between cell and substratum (and minimizing the interaction with the aqueous phase). However, recent electron micrographs of *P. gingivalis* adhering to hexadecane show no such deformations.[45] Another explanation involves the possible cooperative nature of adhesion to hydrocarbons. In some instances, bacteria adhere to hexadecane in lattices which may reflect extensive cell:cell interactions. In some photomicrographs of *P. gingivalis* cells adhering to hexadecane, the adhering cells appear to girdle and stabilize the droplets.[45] Are such cell:cell interactions initiated on the oil:water interface, following adhesion of individual cells and subsequent lateral movement on the interface,[1] or do they adhere preferentially at sites adjacent to already-bound cells (positive cooperativity)? Based on the above, one might predict that certain classes of mutants, isolated by their inability to adhere to hydrocarbons, may actually be capable of interacting with the oil droplets, but unable to bind in a stable fashion, due to deficient ability to interact with other cells.

One central question involves an apparent paradox. Most of the cells which we often refer to as being "hydrophobic" appear to be simultaneously "hydrophobic and hydrophilic". They

tend to adhere to low energy surfaces, yet are wetted by the bulk aqueous phase to the extent that most strains remain suspended over long periods of time in buffer, without aggregating. Why is spontaneous autoaggregation of such strains the exception rather than the rule? If MATH is promoted by cell:cell interactions, why do such interactions appear to predominate on the oil:water interface rather than in the bulk aqueous phase?

The simplest explanation for these phenomena is that of "dynamic hydrophobicity". According to this model, the external configuration of the cell surface components of such strains can undergo dramatic changes. When the cells are suspended in the bulk aqueous phase, the cell surface components and structures assume configurations that maximize interaction with the polar water molecules. Only when such cells come into contact with surfaces (solid surfaces, oil droplets, or one another) are alternate, more hydrophobic structures assumed. For example, adhesive fimbriae might undergo changes in their direction, orientation, degree of bundling, bending, etc. when an interface is encountered. This model would predict that strains which are adherent, but tend to aggregate spontaneously, are less capable of dynamic hydrophobicity (i.e., fixed in a more apolar configuration) than adherent but more suspendable strains such as *A. calcoaceticus* RAG-1 and *S. marcescens* RZ. Thus, microorganisms which appear to adhere similarly to hydrocarbon may differ in their dynamic hydrophobicity, and thus their tendency toward autoaggregation.[45] This phenomenon may similarly account for the observation that some strains appear "hydrophobic" in a particular assay, but "nonhydrophobic" in another.[19,38,89]

In light of the above example, as well as the general confusion surrounding a precise definition of hydrophobicity in general,[90-94] and microbial cell surface hydrophobicity in particular,[19,20,90] it may be prudent to reevaluate our terminology and thinking regarding so-called "cell-surface hydrophobicity assays".

Hydrophobicity is a term which is generally used to account for "unexpected" aspects concerning the behavior of nonpolar entities in water.[90] For example, experiments show that introduction of nonpolar molecules into water is "surprisingly" energetically unfavorable.[93] One model suggests that layers of ordered water molecules form around the nonpolar solute molecule as if it were a "cavity" in the bulk aqueous phase.[91] Such ordered water molecules are more constrained compared to their relative freedom in the bulk aqueous phase, leading to a decrease in the entropy of the system.[93] The observation that the hydrophobic effect increases with temperature, but that this dependence withers as the temperature increases, suggests that these ordered water structures tend to "melt" at higher temperatures, mitigating the entropy effect.[93] Some investigators argue that this temperature dependence is the essence of the "hydrophobic effect".[94]

Presumably, the driving force which enables the hydrophobic interaction between microorganisms and surfaces involves the process reverse to that described above, i.e., the release of energetically "uncomfortable" water molecules from apolar moieties on the microbial surface, as well as the substratum.[20] In such a case, the increase in entropy resulting from this exclusion of water molecules would lead to a decrease in the free energy (since $\Delta G = \Delta H - T\Delta S$).[90] Recent experiments have measured the energy of interaction of two smooth hydrophobic surfaces at molecular distances.[92] Data obtained using this model suggest that hydrophobic interactions are more intense, and of longer range, than was previously assumed.[92] This experimental model appears to be more relevant for understanding the interaction of microbial surfaces with hydrophobic substrata. Nevertheless, there are several important differences. For example, the microbial cell surfaces in question are amphipathic, containing proximal polar and apolar groups, complicating any mechanistic analysis.[91] Furthermore, we have little information on how close to the substratum adhering microorganisms really are.

It must be kept in mind that, whereas some degree of consensus exists regarding some aspects of the hydrophobic effect, there is still considerable controversy over how to define and measure hydrophobicity (this is extremely pronounced in the case of protein conformation and stability).[19] As Duncan-Hewitt points out, we invoke the term hydrophobicity to describe anomolous properties of nonpolar moieties in water

which are poorly understood; had we clear-cut mathematical answers, the term hydrophobicity might become largely superfluous.[90]

Based on the above, it is clear that no single technique can purport to measure the hydrophobicity of microbial cells. Nevertheless, some investigators have gone so far as to suggest a "gold standard" for measuring microbial hydrophobicity.[95] On the contrary, the plurality of assays (and results) helps us understand the complexity of the issues involved. At least for the present, MATH should be taken at face value, i.e., a measurement of the tendency of microorganisms to bind (adhere/adsorb/become trapped) at the interface of water and liquid hydrocarbon. It is clear that microorganisms which do adhere in this assay often tend to adhere to various solid surfaces of interest.[43] A better understanding of the underlying mechanisms of this assay, and its similarities to and dissimilarities from other tests, is needed.

At present, many potential applications of microbial adhesion to hydrocarbons have only been superficially addressed. Would immobilization of cells on hydrocarbon droplets constitute a commercially feasible alternative to current immobilization techniques? Would hydrocarbon-degraders with adhesion capabilities be more efficient in mopping up oil spills than nonadherent strains? Can the lessons of MATH be extended beyond microorganisms to the study of cell organelles, individual molecules, and even inert particles?[96] Does the observed adhesion of so many pathogenic microorganisms to hydrocarbons signify a common medical principle, or suggest a treatment strategy? Hopefully, some of these basic and applied areas of MATH will be developed in this decade.

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BACTERIAL DESORPTION BY COMMERCIAL MOUTHWASHES *vs* TWO-PHASE OIL:WATER FORMULATIONS

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During the past eight years, various investigators have shown that oral microorganisms tend to have hydrophobic surface properties. Most oral microorganisms isolated directly from the oral cavity, as well as many oral laboratory strains, adhere to oil droplets. The authors have recently shown that certain concentrations of the cationic antibacterial agents, i.e. cetylpyridinium chloride (CPC) and chlorhexidine (CHX) promote binding of oral and other microorganisms to oil droplets. This has resulted in development of novel aqueous:oil two-phase mouthwashes containing CPC and/or CHX. In the present manuscript, two such formulations were compared with other commercial mouthwashes for their ability to desorb microorganisms from a solid surface (polystyrene). Of the various mouthrinses tested, only the oil:water formulations efficiently removed the adherent bacterial layer. The data suggest that oil:water mouthwashes may have desorption properties superior to those of many commercial mouthwashes.

KEY WORDS: Adhesion, desorption, mouthwash, polystyrene, oil

INTRODUCTION

Bacterial adhesion to oral surfaces is considered a prerequisite for subsequent colonization (Gibbons, 1984). Development of biofilms on soft (mucosal) oral surfaces is kept in check by continual desquamation of epithelial cells, together with their attached microbiota. However, bacterial accumulation on teeth quickly reaches macroscopic proportions (i.e. dental plaque).

One conceivable approach to limiting bacterial film formation in the oral cavity is to inhibit the initial adhesion process. Indeed, addition of certain sugars, surfactants and chaotropic agents can block adhesion of oral microorganisms to model surfaces, and to one another (McIntire *et al.*, 1982; Nesbitt *et al.*, 1982; E Rosenberg *et al.*, 1983). However, such agents are relatively ineffective in removing bacteria which are already attached. Methods of removing bound microorganisms thus appear to have greater potential. Whereas many studies have been dedicated to investigation of the *in vitro* antimicrobial effects of mouthwashes (Perdok *et al.*, 1989), few, if any, reports exist regarding their bacterial desorption properties.

In 1983, desorption of *Acinetobacter calcoaceticus* RAG-1 cells from polystyrene was reported, using simple oil:saline combinations (Rosenberg *et al.*, 1983). The authors have recently presented evidence that addition of certain cationic antibacterial agents (cetylpyridinium chloride [CPC] and chlorhexidine [CHX]) can greatly increase

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adhesion of microorganisms and oral debris to oil droplets (Goldberg *et al.*, 1990). T... led to the premise that oil:water mouthrinse formulations containing such agents co... serve as effective desorbing mouthrinses, with additional antimicrobial properties.

In the present manuscript, the *in vitro* bacterial desorption ability of two such o... water formulations is compared with that of commercially available mouthrins... using a modified *in vitro* technique (Rosenberg *et al.*, 1983).

MATERIALS AND METHODS

Acinetobacter calcoaceticus RAG-1 (ATCC 31012) cells were grown in brain hear... infusion broth (Difco Ltd) to stationary phase (18 h) with shaking. Cells were har... vested, washed twice and suspended in phosphate buffer (22·2 g $K_2HP0_4 \cdot 3H_2O$, 7·26 ... KH_2PO_4 and distilled water to 1 l, pH 7·1).

One ml of a very turbid suspension of RAG-1 cells (O.D. = 9·3, 400 nm) was added to... disposable polystyrene cuvettes, 1 cm square (4 ml capacity). Following vortexing for 1... min, the RAG-1 cells were poured off, leaving a film of adherent cells. The cuvettes were... washed briefly with tap water. To each cuvette, 1 ml of mouthwash or water (control)... was added (Fig. 1) and the cuvettes vortexed for 2 min. The mouthwash was poured off,... 1 ml fresh mouthwash was added, and the cuvettes again vortexed for 2 min. The... mouthwash was poured off, and the remaining bacterial film stained by adding gentian... violet to the cuvettes for 10 min, followed by washing with water to remove excess stain.... The remaining bacterial film was quantified by measuring the optical absorbance... (585 nm) from both sides of the dried cuvettes in a Uvikon 710 spectrophotometer... (Kontron, Zürich, Switzerland).

Results are presented as the average percent decrease in optical absorbance as... compared to control cuvettes rinsed with water alone. The types of commercial... mouthrinses used in the study, and their origin are presented in Table 1.

Odex is a propriety formulation provided by Agis Ltd consisting of an aqueous... solution containing CPC, sweetener, and food colour, and an oil phase (15% v/v)... containing a mixture of vegetable and essential oils. Odex+ contains, in addition,... 0·025% chlorhexidine gluconate.

RESULTS

In the technique employed, polystyrene cuvettes are first coated with an adherent... layer of *Acinetobacter calcoaceticus* RAG-1 cells. Adhesion of this microorganism to... surfaces has been extensively studied (Rosenberg & Kjelleberg, 1986), and its avid... adhesion to polystyrene has previously been demonstrated (Rosenberg, 1981;... Rosenberg *et al.*, 1983). In the original technique, following desorption by oil:water... mixtures, outgrowth of RAG-1 cells was used to quantify the residual layer of adherent... microorganisms (Rosenberg *et al.*, 1983). Since the present formulations contain anti-... bacterial agents, this approach was modified by using a staining procedure to measure... residual adherent cells. This, in turn, entailed employing a much more turbid cell... suspension in order to obtain a thick film of adherent microorganisms. Quantitation of... desorption could then be ascertained spectrophotometrically.

Results of a typical experiment are summarized in Figure 2 and Table 2. Among the... 12 mouthwashes tested, only Odex and Odex+ were highly effective in removing the... dense bacterial film, desorbing 98% and 92%, respectively, as compared to the control...

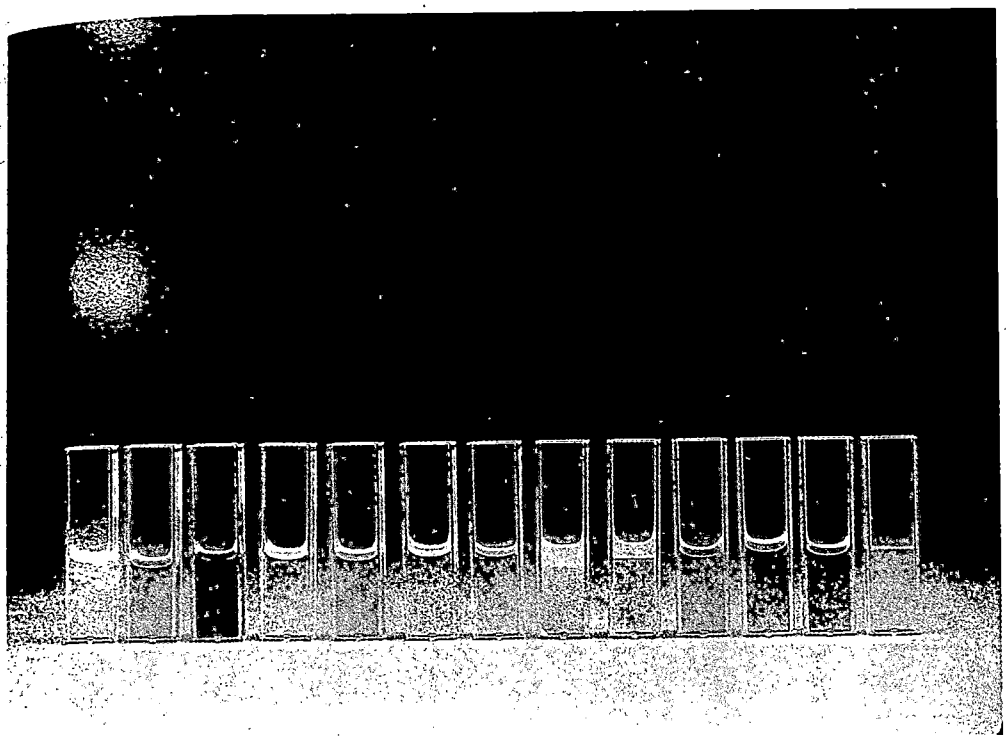

Fig. 1 Polystyrene cuvettes containing the mouthwashes, prior to mixing. From left to right: Listerine®, Eludril, Act®, Cepacol®, Listermint™, Scope®, Corsodyl™, Odex, Odex+, Colgate® Tartar Control Mouthwash, Veadent®, Signal®, water. (See colour plate).

cuvettes rinsed in water alone. Other commercial mouthrinses tested removed between 15% (Corsodyl ™) and 50% (Listerine®) of the bacterial film. Desorption by both oil: water formulations was significantly greater ($P < 0{\cdot}005$) than that observed for all the other mouthwashes.

DISCUSSION

The present report shows that oil:water formulations containing CPC or CPC and CHX, have the capacity to desorb large proportions of bound microorganisms from surfaces as compared to conventional mouthwashes. It has been previously shown (Rosenberg *et al.*, 1983) that aqueous:olive combinations removed over 97% of bacterial film from polystyrene cuvettes, compared to control cuvettes rinsed with water alone. In that study, desorption was measured by subsequent outgrowth of the remaining cell layer. However, since commercial mouthrinses routinely contain antibacterial agents, the outgrowth approach precluded comparison of the desorption efficacy of oil: water mixtures with commercial preparations. Furthermore, a much higher initial concentration of cells was used in the present study, resulting in a more recalcitrant biofilm. Finally, the presence of the cationic antibacterial surface active agents, cetylpyridinium chloride and chlorhexidine, in the oil:water formulations did not

Table 1 Mouthrinses Used in This Study

Name of Mouthwash	*Manufacturer*
Listerine®	Warner-Lambert Co., Morris Plains, NJ 67950
Eludril	PF Medicament, 125, rue de la Faisanderie, Paris, France
Act®	Johnson & Johnson, New Brunswick, NJ 08903
Cepacol®	Merrell Dow Pharmaceuticals Inc, Cincinnati, Ohio 45215
Listermint (trade mark)	Warner Lambert Health Care, Eastleigh, Hants, England
Scope® (Peppermint)	Proctor & Gamble, Cincinnati, Ohio 54202
Corsodyl (trademark)	Imperial Chemical Industries, PLC, Macclesfield, Cheshire, Great Britain
Odex	Agis Ltd, Tel-Aviv
Odex +	Agis Ltd, Tel-Aviv
Colgate® Tartar Control Mouthwash	Colgate Palmolive Co., NY, NY 10022
Veadent®	Glaxo (under license from Vipont Laboratories Inc), Fl. Collins, USA
Signal®	Lever Brothers Co., 390 Park Avenue, NY, NY 10022

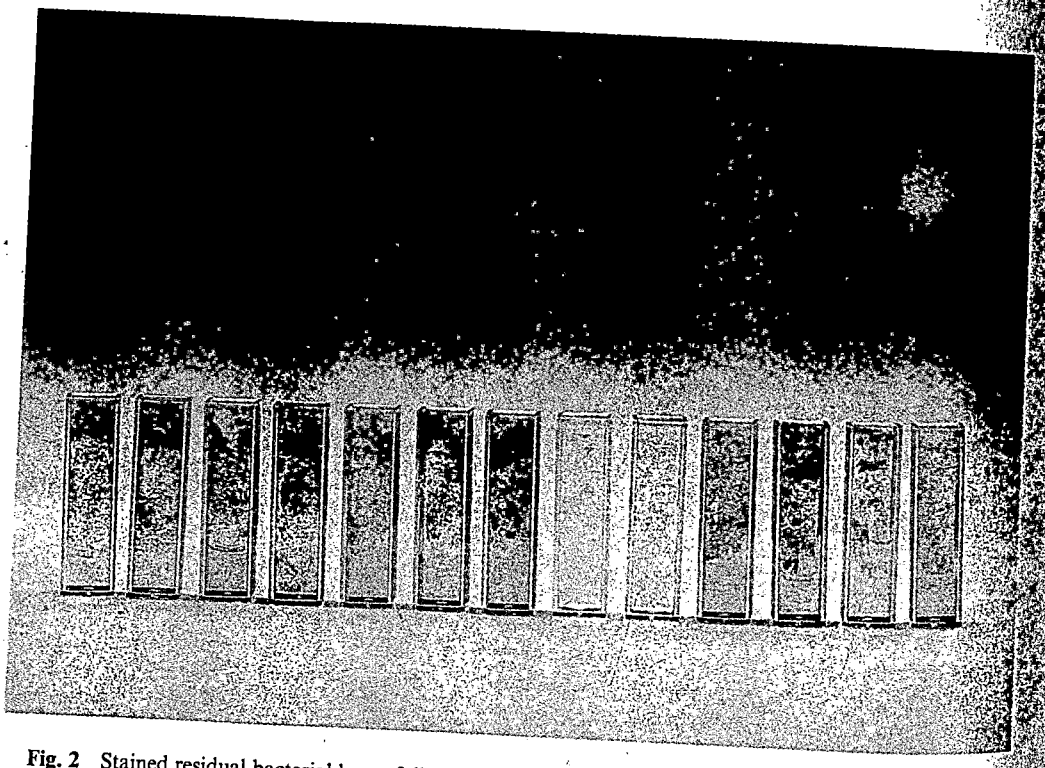

Fig. 2 Stained residual bacterial layer, following rinsing procedure. Cuvettes were layered with bacterial film, washed, rinsed with mouthwashes and stained as described in Materials and Methods. Order of cuvettes is the same as Figure 1. (See colour plate).

Table 2 Desorption of an Adherent Bacterial Film by Mouthwashes

Name of Mouthwash	*Percent Desorption compared to water control*[a]
Listerine®	50
Eludril	34
Act®	40
Cepacol®	40
Listermint (trademark)	36
Scope® (Peppermint)	28
Corsodyl (trademark)	15
Odex	98
Odex+	92
Colgate® Tartar Control Mouthwash	21
Veadent®	28
Signal	44

[a]Average of 2 readings

adversely affect the desorption properties. Addition of either of these agents to oil: water mixtures results in long-term reductions in oral malodour and microbial levels (Y Konis & M Rosenberg, unpublished). It should be emphasized that commercial mouthrinses containing cetylpyridinium chloride (e.g. Cepacol®, Scope®), or chlorhexidine (e.g. Eludril, Corsodyl (trademark)) were relatively ineffective in desorbing the bacterial layer. Indeed, Odex preparations are relatively ineffective desorption agents when the oil phase is excluded.

The present study is based on an *in vitro* technique based on desorption from a smooth, hydrophobic surface. Whereas hydrophobic interactions are important for oral adhesion, they probably act in concert with other mechanisms. Nevertheless, hydrophobic inhibitors are able to block adhesion to oral surfaces and to desorb adherent cells (Nesbitt *et al.*, 1982; Rosenberg and Kjelleberg, 1986; E Rosenberg *et al.*, 1983). Moreover, whereas the well-characterized model organism employed here (RAG-1) is not an oral microorganism, ODEX also effectively desorbs *Actinomyces viscosus* and *Streptococcus sanguis* from polystyrene tubes (not shown). Furthermore, microscopic observation of ODEX following intraoral use shows that high levels of oral microorganisms are concentrated on the surface of expectorated oil droplets (Rosenberg, 1990*b*).

Finally, *in vitro* experiments indicate that rinsing with ODEX removes more stainable oral debris than rinsing with the aqueous phase of ODEX alone. These findings suggest that ODEX also has *in vivo* oral desorption capabilities and that the oil phase is critical for the desorbing effect.

The above notwithstanding, it is likely that efficient bacterial desorption is only one of several criteria to be considered when discussing mouthwash efficacy. For example, whereas Corsodyl™, a chlorhexidine mouthrinse, is highly effective in reduction of microbial levels (Perdok *et al.*, 1989) and oral malodour (Rosenberg, 1990*a*), in the

present report, it exhibited the poorest desorption capacity among all mouthwashes tested. We can consider the following hypothetical example: a nonbacteriostatic, nonbactericidal mouthwash that desorbs 75% of oral microorganisms (a daunting challenge, considering the concentration of oral microorganisms in multilayered plaque and sequestered subgingival sites) can be effective for only two generations, since only two doublings are required to regain original microbial levels. However, a mouthwash that desorbs 75% of the oral microbiota and kills 75% of the residual microorganisms would be three times as effective. Thus, a formulation such as ODEX+, which is still an effective desorber (92%), yet contains a cocktail of antibacterials, including CHX, may have high efficacy. *In vivo* experiments to test the long-range effects of such oil:water formulations on oral microbial levels and general oral hygiene and health are thus warranted.

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This Week's Citation Classic ®

Rosenberg M, Gutnick D & Rosenberg E. Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. *FEMS Microbiol. Lett.* 9:29-33, 1980.
[Dept. Microbiology, George S. Wise Fac. Life Sciences, Tel Aviv Univ., Ramat Aviv, Israel]

A simple method is proposed for measurement of cell-surface hydrophobicity of microorganisms. Turbid, washed cell suspension (1.2 ml) is vortexed for two minutes in the presence of 0.01-0.2 ml of test hydrocarbon (*n*-hexadecane, *n*-octane, and *p*-xylene). Adhesion is calculated from the decrease in turbidity of the lower aqueous phase. [The *SCI* ® indicates that this paper has been cited in more than 250 publications.]

Mixing Water, Oil, and Microorganisms

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In 1971, Eugene Rosenberg and David Gutnick initiated a highly successful collaboration in petroleum microbiology at Tel Aviv University that would last for some 15 years. When I joined Eugene's lab as a PhD candidate in the fall of 1978, the "oil club" was at its zenith, with some 15 postdoctoral fellows, graduate students, and technicians.

My initial interest was in studying adhesion of oral microorganisms. E. Rosenberg managed to persuade me that unraveling the secrets of how hydrocarbon-degrading bacteria adhered to oil droplets would eventually have some bearing on the oral cavity. I was subsequently assigned to study the mechanism responsible for the observed adhesion of *Acinetobacter calcoaceticus* RAG-1 to hydrocarbon and its role in enabling growth on this water-insoluble substrate.

The technique described in this *Citation Classic* came about via a circuitous route. I had been trying to generate uniform coatings of solid paraffin on various surfaces and subsequently measure adhesion. David, having read a recent article by M. Fletcher and G.I. Loeb,[1] suggested that polystyrene might be a suitable surrogate attachment surface. A strain of *Serratia marcescens* adhered so magnificently to polystyrene that we were tempted to test its (heretofore unreported) ability to degrade hydrocarbons. I prepared a minimal growth medium containing phosphate, urea, and magnesium sulphate (so-called PUM buffer), overlaid with n-hexadecane. Anxious to see any net increase in cell growth, I added a particularly turbid inoculum of cells and aerated overnight with vigorous shaking. The next morning I was perplexed to find that not only had the cells *not* grown, but they appeared to have disappeared altogether! They turned up after a brief search, bound to the surface of the hexadecane droplets.

It soon became evident that a short duration of strong vortexing was sufficient to distinguish between cell suspensions that adhered and those that did not. The extent of adhesion to increasing volumes of several test hydrocarbons could be simply ascertained by measuring the drop in turbidity in the lower aqueous phase following phase separation.

Initially disappointed that adhesion to hydrocarbon was not unique to oil-degrading microorganisms, it slowly dawned on us that we had discovered a much more general phenomenon. As it turned out, we were not the first to link microbial adhesion to oil with cell-surface hydrophobicity.[2,3] However, a fortuitous choice of adherent and nonadherent strains, together with the demonstration that surfactant reversed the adhesion, supported the premise that the hydrophobic surface properties of a wide variety of microbial cells could be measured in this extremely simple manner.

Since other hydrophobicity techniques were already available for studying bacterial surfaces, we did not initially anticipate that the assay would generate much interest. However, soon after the paper appeared, the technique was successfully used by several leading adhesion researchers, including I. Ofek, E. Beachey, R. Doyle, and R.J. Gibbons. This probably was an important factor in establishing the assay.

The bacterial adhesion to hydrocarbons (BATH) test, in various modifications, is still routinely employed (since it is also used to study fungi, it has been recently renamed the "microbial adhesion to hydrocarbons," or MATH, test).

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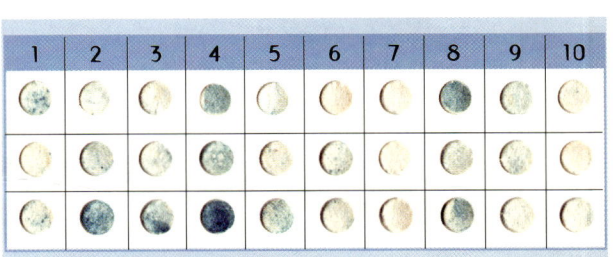
ריחות פה - איבחון זטיפול

פרופ' מל רוזנברג .Ph.D*

תופעת ה: "**Bad Breath Paradox**" מסבירה כיצד ישנם מיליוני אנשים וביניהם רופאי שיניים אשר סובלים מריח פה חריף ואינם מודעים לכך כלל. בשנת 1993 כינתי את הקבוצה השניה "הליטופובים" (דהיינו - פוביה מהליטוזיס) היום המונח מקובל בספרות המקצועית לתאור אותם פציינטים המגזימים בצורה קיצונית בהתייחסותם לנושא.

ההתעניינות שלנו בנושא ריח פה החלה בתחילת שנות השמונים. יחד עם ד"ר אירוין וייס פיתחנו מי-פה דו - פאזיים המכילים מים ושמן וגילינו שטיפות השמן מסוגלות לקשור חיידקים ולהסירם מהפה ביעילות רבה. הצגנו את ההמצאה לפני חברת טבע ב-1983 הם הציעו לנו לבדוק כיצד התערובת פועלת למניעת ריחות פה.
כאשר פנינו לספרות המקצועית מצאנו להפתעתנו שיחסית מעט היה ידוע על ריחות פה. זאת מפני ששיטות המדידה היו סבוכות. במשך חמש עשרה שנה עבדנו על פיתוח שיטות מהירות וכמותיות היכולות לעזור לקלינאי לבדוק את הנושא וגם לאפשר מחקר קליני אודות ריחות פה. ב-1986 הצענו לראשונה שימוש במוניטור נייד לניטור גופרית מחוזרת כשיטה לבדיקה מהירה ופשוטה הקשורה לריח פה. כיום שיטה זו מקובלת בעבודות מחקר באקדמיה ובתעשיה וכן בלמעלה מאלף מרפאות שיניים ברחבי העולם. בימים אלו אנחנו שוקדים על פיתוח בדיקה פשוטה וזולה לבדיקת ריחות פה. בדיקה הנקראת בלעז **Ok2kiss** (אפשר להתנשק) מבוססת על נייר הספוג בחומר שמשנה את צבעו לכחול בנוכחות אנזימים מהרוק הקשורים להתפתחות ריח פה. על מנת להפעילה יש לשים טיפת רוק על הנייר והופעת צבע כחול תוך מספר דקות מעידה על הצורך לנקוט פעולות מנע. את הבדיקה מפתח במעבדתנו ד"ר ניר שטרר.



תמונה 1

בכל העולם מוצרים למניעת ריח בפה מכילים מנטה בתור חומר טעם, זו על פי התפיסה בציבור שמנטה מפיגה ריחות פה. למעשה פעילותה האנטיבקטריאלית של המנטה היא מוגבלת וכן יעילותה. בשנה האחרונה, במסגרת חממת רד-רמות, פותח חומר טעם חדש הנקרא Breathanol אשר לו פעילות מוגברת למניעת ריח פה. בניסוי קליני שבו השתתפו 54 משתתפים שנערך באוניברסיטת תל-אביב הודגמה יעילותו של ה-Breathanol. היות וה-Breathanol הוא חומר טעם אכיל, ניתן להוסיפו למגוון רחב של מוצרים כגון סוכריות, מסטיק, מי-פה, טיפות פה וכ"ו. משחת שיניים להפגת ריח פה המכילה Breathanol יוצאת לשוק המקומי בימים אלה ממש.

ממה נובע ריח פה?

רוב הריחות הדוחים אותנו בסביבתנו הקרובה נובעים מפעילות חיידקים. ריח גוף, ריח צואה, ריח זבל וריח ביוב הם רק כמה דוגמאות. קיימת דעה רווחת בציבור שריח פה מקורו בקיבה. רב החוקרים מסכימים שריח רע שמקורו בקיבה הוא נדיר ביותר. לעומת זאת, יש ראיות שב-85% עד 90% מהמקרים אפשר לייחס את הריח הרע לפעילות מיקרוביולוגית בתוך חלל הפה עצמו. בפה הריח מתחיל מפירוק של חלבונים בתהליך אנאירובי. תוצרי הפירוק - חומצות האמינו, ממשיכים להתפרק ע"י החיידקים בפה וחלק מתוצרי הפירוק הם בעלי ריח רע חזק. דוגמאות לכך כוללות: H_2S מציסטאין, מתיל מרקפטן ממתיונין, אינדול וסקטול מטריפטופאן, קדברין מליזין ואורניתין. לכן למוצרים אנטיבקטריאליים יש פעילות למניעת ריח פה. צריך להדגיש שהיות וחלק גדול מהפעילות החיידקית הנ"ל נעשית בחלק האחורי של גב הלשון, השימוש במי פה הוא לא אפקטיבי ללא גירגור המפגיש את החומר האנטיבקטריאלי עם החיידקים שבחלק האחורי של גב הלשון.

תמונה 2

אפשר למנות מספר גורמים אשר מגבירים את הסיכוי לריח פה: הראשון הוא הגיינה אורלית לקויה במיוחד באתרים אינטרפרוקסימלים. במחקר שפרסמו קוזלובסקי ושות' ב-1994 הייתה קורלציה גבוהה בין פלאק אינדקס לבין עוצמת הריח ב - 52 נבדקים ישראליים. מעבר לכך בנבדקים שהשתמשו בחוט דנטלי ניתן היה לחוש פחות ריח בצורה מובהקת.

• פרופסור מן המנין למיקרוביולוגיה, מיקרוביולוגיה הזמנית הפקולטה לרפואה ע"ש סאקלר, ביולוגיה אורלית ביה"ס לרפואת שיניים ע"ש מורים וגבריאלה גולדשלגר, אוניברסיטת תל אביב.

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Reproducibility and Sensitivity of Oral Malodor Measurements with a Portable Sulphide Monitor

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Forty-one subjects with bad breath were assessed for oral malodor and periodontal status on three occasions, at intervals of approximately one week. Oral malodor was assessed by measurement of peak and steady-state volatile sulphide levels with a portable sulphide monitor and by organoleptic measurement of whole-mouth, tongue dorsum, and interproximal dental odors by two independent judges. Reproducibility of measurements, assessed by paired *t* tests and Kappa testing, demonstrated no significant differences between any of the test results from the first and second appointments. Steady-state sulphide levels were the most reproducible of all tests. The ability of the tests to detect an expected reduction of malodor following a 0.2% chlorhexidine mouthrinse regimen was investigated by comparison of test values between the second and third appointments. Following the mouthrinsing treatment, 43% reductions of peak, 47% reductions of steady-state volatile sulphide levels, and 15-58% reductions in all other measurement categories were observed. The majority of the participants (22/41) had no pockets greater than 5 mm and exhibited both moderate gingival inflammation (Mean Gingival Index = 1.17) and moderate plaque accumulation (Mean Plaque Index = 1.84). Plaque Index and presence of pockets greater than 7 mm were weakly related to sulphide measurements. Whereas assessment of steady-state sulphide levels by the sulphide monitor does not constitute a direct measure of oral malodor, its relation to organoleptic measurement, superior reproducibility, objectivity, and sensitivity support the use of the sulphide monitor in clinical studies.

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Introduction.

Bad breath (halitosis, oral malodor, *fetor ex ore*) is a common human condition, affecting primarily the adult population (Tonzetich, 1977). Most cases of bad breath originate from microbial putrefaction within the oral cavity itself, and volatile sulphide compounds have been cited as the predominant source of foul odor (Tonzetich, 1978; Kleinberg and Westbay, 1990). In addition to the social aspects of oral malodor, measurement of bad breath may provide a useful screening test for detection of periodontal diseases (Tonzetich, 1978) on the basis of the capacity of subgingival microbiota to produce hydrogen sulphide (Persson *et al.*, 1989).

For the assessment of oral malodor in scientific investigations, reliable methods of measurement are required. Previous published methods of assessing oral malodor have been based upon subjective, organoleptic (hedonic) scales (Nara, 1977; Tsunoda *et al.*, 1981; Ishikawa *et al.*, 1984), or quantitative measurement of volatile sulphide compounds (VSC; Kostelc *et al.*, 1980, 1984), or both organoleptic and VSC measurements (Schmidt *et al.*, 1978). Quantitative measurements by gas chromatography (Solis-Gaffar *et al.*, 1975; Tonzetich, 1977; Kostelc *et al.*, 1984) cryo-osmoscopy (Brening *et al.*, 1939), and culture of plaque and periodontal pocket exudates in bacteriological media (Tachibana, 1957) require elaborate and sophisticated laboratory facilities, and may be time-consuming and expensive. Limited data are available on the reproducibility of such measurements, which may be confounded by circadian variation, menstrual cycle, smoking habits, variations in oral hygiene, climate, and other variables. Moreover, once the gases in the oral cavity are evacuated for measurement, the kinetics of re-establishment to previous levels or proportions is unknown.

Recently, a simple and rapid technique for measurement of volatile sulphide concentration by use of a portable instrument has been proposed (Rosenberg *et al.*, 1991). The aim of the present report was to compare the reproducibility and sensitivity of organoleptic methods for oral malodor assessment with those of the sulphide monitor.

Materials and methods.

Patient population.—Subjects were chosen from among approximately 200 people who responded to an article on bad breath that appeared in a local newspaper. Letters explaining the project were distributed to all respondents, and the first 42 subjects who agreed to participate were enrolled. All subjects reported subjective complaints of bad breath. Two subjects who took antibiotics within two weeks prior to their appointments were deferred for two months prior to starting. There were no exclusion criteria. The total duration of the study was 12 weeks.

Subject preparation.—Subjects were asked to refrain from oral activities, including eating, drinking, chewing, sucking, and mouthrinsing, for at least two hours prior to each appointment. For assessment of reproducibility, measurements were made at the first and second appointments, which were held at intervals of approximately one week. For assessment of the sensitivity of the measurement methods in detecting expected changes in bad breath, patients were instructed to rinse twice daily with a 0.2% chlorhexidine (CHX) gluconate mouthrinse (Contrex Super, Microbex, Markham, ON, Canada). Patients rinsed according to manufacturer's instructions, immediately before bedtime and in the early morning. Each treatment consisted of two consecutive 30-second rinses, with no eating or drinking for at least 30 min afterward. Rinsing was performed over a one-week period from the evening following the second appointment to the evening preceding the third and final appointment.

Volatile sulphide concentration.—VSC measurements were made with a portable industrial sulphide monitor (model 1170, 0.5 ppm, full-scale, Interscan Corp., Chatsworth, CA), zeroed on ambient air before each measurement. A disposable 6.5-mm plastic straw was attached to the air inlet of the monitor. Each patient was instructed to bring his slightly-opened mouth over the straw so that it extended approximately 4 cm into the oral cavity , and to breathe through his nose during the measurement. This instrument contained a pump which sucked air from the plastic straw placed inside the patient's mouth at 1500 mL/min. The sample of mouth air passed through a sensor, which registered the level of sulphides. Both peak (maxi-

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mum) and steady-state levels attained were determined in parts *per* billion (ppb) sulphide equivalents by direct readings from the analog scale of the monitor.

Organoleptic assessment.—Organoleptic assessment was made by two judges, as previously described (Tonzetich and Ng, 1976; Rosenberg *et al.*, 1991). One of the judges had previous experience in measuring several hundred subjects (M.R.). The second judge (A.B.) was an experienced dental hygienist who had not participated previously in organoleptic assessment of malodor. The judges were not pre-calibrated. Organoleptic malodor scores were recorded independently by each judge on a scale of 0 to 5 as follows: 0 - no appreciable odor; 1 - barely noticeable odor; 2 - slight, but clearly noticeable odor; 3 - moderate odor; 4 - strong odor; and 5 - extremely foul odor. Judges were blinded regarding one another's scores, their own previous scores, and VSC measurements. Patients were instructed not to speak for several minutes prior to bad breath measurement.

Tongue malodor was assessed by one of the judges (A.B.) by scraping the dorsum of each patient's tongue with a gauze pad and immediately smelling its odor. Floss malodor was assessed by the passing of waxed dental floss interproximally between all posterior teeth and assessment of the odor following each interproximal passage, based on the above organoleptic scale. Floss was similarly passed between all anterior teeth, but the odor was assessed after passage between all maxillary and mandibular anterior teeth. Different segments of floss were used for each interproximal passage. The highest level of malodor, based on the above organoleptic scale, was recorded.

Measurements of Plaque Index (PI; Silness and Löe, 1964) and Gingival Index (Löe and Silness, 1963) were performed on all teeth at each appointment following malodor measurements. The depth of periodontal pockets was measured with a Michigan O probe (HuFriedy, Chicago, IL) to the nearest mm marking. Unstimulated saliva was collected and scored as present if it exceeded 0.2 mL over a two-minute period.

Statistical analysis.—The reproducibility of the VSC data was assessed by means of a paired *t* test and by plots of differences against means. Intra- and inter-examiner consistencies were assessed by Cohen's Unweighted Kappa Statistic (Cohen, 1960). The sensitivity of the tests in detecting an expected reduction in test values after CHX was assessed by the paired *t* test. The Pearson correlation was used to assess the strength of association between various malodor measurements and periodontal parameters. Multiple regression analysis with the GLM procedure of SAS (SAS 6.03, Cary, NC) was used to examine the relation among steady-state VSC measurements, organoleptic scores, and other patient variables. All analyses were performed with SAS software (SAS 6.03, Cary, NC).

Results.

Forty-one subjects participated in this study, of whom 14 were male (47.0 ± 15.3 years; $\bar{X}$ ± S.D.) and 28 were female (52.3 ± 12.2 years). One person failed to complete the study.

Transformation of peak and steady-state sulphide measurements to natural logarithms (LnPk and LnSs, respectively) yielded near-normal distributions which were checked by means of the univariate procedure and the Shapiro-Wilk test in SAS (Shapiro and Wilk, 1965). Since the possibility existed that the sensitivity of the sulphide sensor decreased with time over the 12-week duration of the study, the decline in the peak and steady-state measurements was estimated between the first and second appointments by computation of the systematic reduction in mean values. Mean peak measurements declined 1.04 ppb/week and mean steady-state measurements by 1.03 ppb/week. Whereas both measurements showed a slight decline, only peak measurements were seen to decline significantly at the 5% level. The finding of declining values

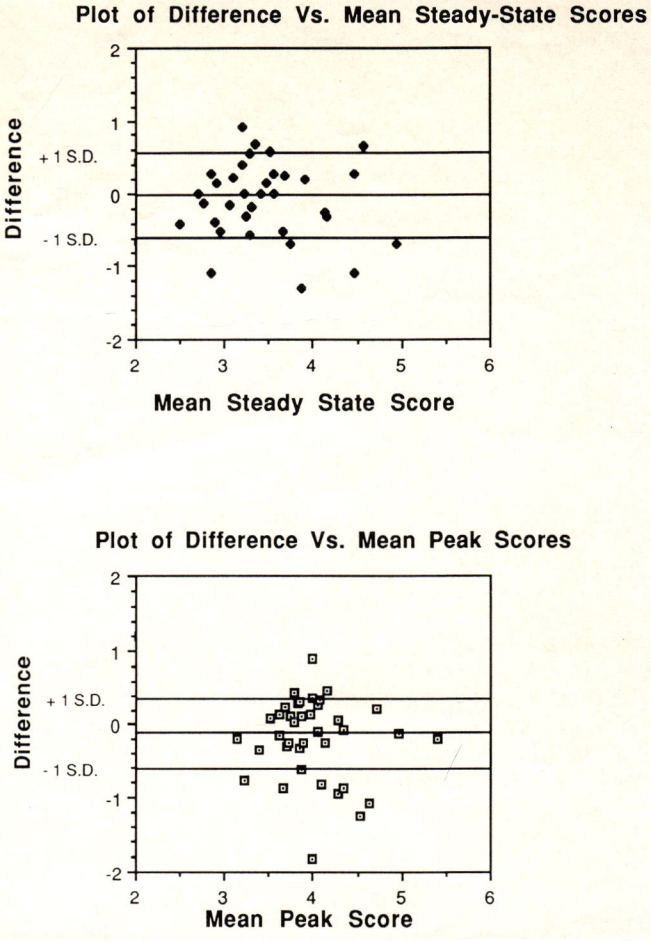

Fig.—Plot of differences between VSC scores at first and second appointments against mean scores for steady-state and peak measurements. Horizontal lines represent one standard deviation from mean. Note that the mean difference is slightly less than zero, indicating either instrument drift or systematic reduction in VSC between first and second appointments.

with time indicated the possibility of declining instrumental sensitivity. Consequently, all measurements were adjusted for these systematic reductions in sulphide measurement.

No significant differences were found in the adjusted peak and steady-state sulphide measurements made at the first and second appointments (Table 1), and this was true for both sexes (paired *t* test; $p > 0.05$). Plots of differences between peak and steady-state measurements at the first two appointments against their respective means demonstrated that, while all of the data points were within two standard deviations of the overall mean difference, 58% of steady-state values and 48% of peak values were within one standard deviation of the mean, demonstrating the superior reproducibility of steady-state values (Fig.).

Intra- and inter-examiner consistencies of whole-mouth organoleptic measurements were assessed by Cohen's Unweighted Kappa statistic, which assesses the frequency with which examiners can exactly replicate either their own scores or the scores of another examiner. The Kappa statistic provides a rigorous test of examiner agreement, and it is suggested (Landis and Koch, 1977) that a score greater than 0.4 indicates moderate agreement. Intra-examiner consistency ($k = 0.38$ for M.R.; $k = 0.32$ for A.B.) was low but was slightly better than inter-examiner consistency ($k = 0.22$). Consistency in the organoleptic tongue and floss scores between the first and second appointments was similarly analyzed and yielded Kappa values of 0.13 and 0.05, respectively. In contrast to Kappa testing, Pearson correlation analysis indicated a reasonably strong associa-

TABLE 1

MALODOR MEASUREMENTS AND DENTAL PARAMETERS

Measurement	Appointment 1	Appointment 2	Appointment 3
Peak VSC	67.2 ± 7.3	56.2 ± 5.3	35.1 ± 3.0
Steady-state VSC	40.7 ± 5.9	35.6 ± 4.1	20.2 ± 3.1
MR-whole mouth	2.5 ± 0.2	2.5 ± 0.2	1.2 ± 0.2
AB-whole mouth	2.4 ± 0.2	2.3 ± 0.2	1.0 ± 0.2
Tongue score	2.5 ± 0.1	2.5 ± 0.1	1.8 ± 0.1
IP score	3.1 ± 0.2	3.1 ± 0.2	2.4 ± 0.2
Plaque Index	1.8 ± 0.1	1.8 ± 0.1	1.4 ± 0.1
Gingival Index	1.2 ± 0.1	1.1 ± 0.1	0.9 ± 0.1
Saliva	27/11	28/11	29/11
Present/Absent	(missing obs. = 3)	(missing obs. = 2)	(missing obs. = 1)

Values represent mean ± SEM.

tion between the two organoleptic examiners ($r = 0.71$; Table 2) and between the organoleptic assessments and steady-state VSC measurements ($r = 0.60$ for M.R.; $r = 0.52$ for A.B.).

Significant reductions of peak (43%; $p = 0.0001$) and steady-state (47%; $p = 0.0001$) sulphide measurements were observed at the third appointment following the CHX mouthrinse treatment. Similarly, whereas there were no significant differences in the organoleptic odor scores from whole-mouth, tongue, and floss between first and second appointments, they were significantly reduced following the CHX mouthrinse. There were 52% and 58% reductions in the organoleptic whole-mouth ratings, as scored by examiners M.R. and A.B., respectively ($p = 0.0001$). Smaller reductions were observed in the assessment of tongue odor (27%; $p = 0.0001$) and floss odor (22%; $p < 0.005$) scores.

Whole-mouth, tongue, and interproximal (floss) odor scores were found to be independent of the presence or absence of saliva (Chi sq.; $p > 0.05$). Similarly, the peak and steady-state VSC scores, gender, and appointment number were independent of the presence or absence of saliva (Chi sq.; $p > 0.05$). Steady-state and peak sulphide measurements as well as organoleptic scores of malodor were positively related (slope = 0.008; $p = 0.09$) to the age of the subject. This age-related increase was similar in the two sexes and was significantly different from zero ($p = 0.09$); however, there was a large scatter of the data ($r = 0.14$).

Multiple regression analysis of log-transformed steady-state and peak VSC measurements and of organoleptic scores demonstrated that whole-mouth organoleptic scores of one examiner (M.R.), PI, and duration in the study period accounted for most of the variation in the VSC measurements. Individual factors—such as age, gender, GI, organoleptic tongue and organoleptic floss scores—were found to account for a smaller portion of the variability in steady-state scores. These weak associations were also demonstrated by the low r values of individual parameters obtained by Pearson correlation analysis (Table 2). However, when taken together, their overall association with VSC measurements was marked when analyzed by multiple regression ($r = 0.67$).

Twenty-two patients had no periodontal pockets deeper than 5 mm, and the other 19 patients had between one and nine pockets deeper than 5 mm. Three patients had one or more 7-mm pockets (*i.e.*, moderate to advanced disease). Therefore, the majority of these patients exhibited gingivitis without periodontitis (22/41). The mean PI for the first two appointments was not significantly different ($p > 0.05$). At the third appointment following CHX, there was a significant reduction in the mean PI score (26%; $p < 0.0001$).

Patients with one or more pockets greater than 5 mm had steady-state VSC scores 30% higher than those of patients without pockets ($p < 0.05$). A single individual with nine pockets > 5 mm was observed to have steady-state and peak scores higher than those of individuals with fewer pockets (data not shown). However, Pearson correlation analysis demonstrated weak associations between the presence of pockets greater than 5 mm and both organoleptic and VSC measurements (Table 2). No strong formal inferences can be drawn about the relationship between number of pockets and malodor scores, because very few subjects demonstrated severe pocketing, and those with pockets exhibited wide variations in the numbers of pockets.

Discussion.

The sulphide monitor detects primarily hydrogen sulphide. Other VSC's, such as dimethyl sulphide and methyl mercaptan, are also detected, but the detection sensitivity of the instrument to these gases is about 50% of that for hydrogen sulphide (data on file, Interscan Corp., Chatsworth, CA). Since VSC's are known to be important contributors to oral malodor (Tonzetich, 1977), measurement of VSC's made with the monitor could provide a simple and objective approach to malodor assessment. In this report, intra-oral sulphide levels made one week apart in individuals with a chief complaint of bad breath were reproducible, thereby demonstrating an essential prerequisite for the rational use of a diagnostic instrument (Sackett *et al.*, 1985). The reproducibility between the first two appointments was surprising, since we anticipated that confounding factors, such as greater attention to oral hygiene, might have resulted in considerably reduced VSC scores at the second appointment. The instrument was also sensitive to reduced VSC levels resulting from CHX treatment. This expected reduction of VSC levels was consistent with previous data after mouthrinse treatment (Schmidt and Tarbet, 1978) and indicates that the sulphide monitor was sufficiently sensitive for detection of reduced levels of sulphide-containing compounds after anti-microbial treatment.

Previous studies have reported significant correlations between peak sulphide measurements with the sulphide monitor and mal-

TABLE 2

PEARSON CORRELATION ANALYSIS OF MALODOR MEASUREMENTS AND DENTAL PARAMETERS

	LnPk	LnSs	MR	AB	Tongue	Floss	PI	GI	P > 5
LnPk	1.00	0.879	0.552	0.425	0.377	0.208	0.373	0.087	0.280
	0.0	0.0001	0.0001	0.0001	0.0001	0.026	0.0001	0.053	0.002
LnSs		1.00	0.599	0.518	0.353	0.250	0.396	0.067	0.257
		0.0	0.001	0.0001	0.0001	0.007	0.0001	0.479	0.006
MR			1.00	0.714	0.408	0.381	0.353	0.099	0.184
			0.0	0.0001	0.0001	0.0001	0.0001	0.292	0.047
AB				1.00	0.406	0.422	0.359	0.081	0.107
				0.0	0.0001	0.0001	0.0001	0.383	0.250
Tongue					1.00	0.237	0.384	0.255	0.091
					0.0	0.01	0.0001	0.006	0.332
Floss						1.00	0.293	0.189	0.224
						0.0	0.001	0.042	0.016
PI							1.00	0.223	0.006
							0.0	0.016	0.945
GI								1.00	0.143
								0.0	0.124

Pearson Correlation Coefficients/Prob> |R| under Ho: Rho = 0.

Data from all three appointments were analyzed together. LnPk - natural logarithm of peak VSC measurement, LnSs - natural logarithm of steady-state VSC measurements, MR - organoleptic examiner, AB - organoleptic examiner, Tongue - organoleptic score of tongue scraping by AB, Floss - organoleptic assessment of floss by AB, PI - Plaque Index, GI - Gingival Index, P > 5 - number of pockets greater than 5 mm.

odor as determined by a seven-member organoleptic panel (Rosenberg *et al.*, 1991). These reported correlations are consistent with previous data describing gas chromatographic measurements of peak VSC and organoleptic assessments of oral malodor (Schmidt *et al.*, 1978). Since peak measurements may depend on the manner in which the sampling straw is introduced into the oral cavity, and may be greatly influenced by the kinds of oral activities carried out prior to testing (*e.g.*, talking, swallowing, mouth breathing, etc.), steady-state values were recorded as well. Indeed, steady-state values (which indicate the on-going rate of sulphide generation within the mouth) were more reproducible and sensitive to CHX treatment than were peak measurements. Mean steady-state values also increased with age, consistent with early data (Sulser *et al.*, 1939).

The measuring instrument used in this study exhibited an apparent slight downward drift with time. This reduction may reflect a systematic decline in machine sensitivity due to contamination of the H_2S sensor with sulphide-containing components. Consequently, the instrument must be re-calibrated periodically to adjust for the loss of sensitivity. The reduction in scores between first and second appointments may reflect real declines in VSC, possibly induced by improved patient oral hygiene during the study. Verification of the source of this decline awaits the use of highly calibrated standards.

In addition to whole-mouth assessment, we also measured malodor from two intra-oral sites considered to be major foci of malodor, *i.e.*, the dorsum of the tongue and the interproximal areas (Spouge, 1964; Tonzetich and Ng, 1976; Tonzetich, 1977). Whereas bad breath was associated with tongue malodor, interproximal odor, or both in specific patients, neither organoleptic parameter was reproducible, and did not correlate well with the other scoring techniques. Thus, the organoleptic rating of material scraped from these sites by a single observer (A.B.) did not appear to make a significant contribution to overall bad breath measurement in patients with a chief complaint of halitosis. This finding is in contrast to the data of Tonzetich and Ng (1976), who found in normal subjects that dorsal tongue surfaces were the source of the most offensive compounds when assessed by a panel of pre-calibrated judges. It is not clear from the present report whether patients who present with a chief complaint of halitosis may represent a unique population of individuals in whom VSC-producing sites are not restricted to the tongue. Further work is now in progress with site-specific sampling of sulphide compounds from tongue and interproximal sites with a modification to the sulphide monitor.

Based on the superior reproducibility and sensitivity of the sulphide measurements compared with organoleptic measurements, the sulphide monitor may be a valuable tool for assessment of oral malodor. The sulphide monitor also presents other advantages such as: (i) no need for skilled personnel; (ii) non-invasive; (iii) low likelihood of cross-infection; (iv) portability; (v) relatively inexpensive; and (vi) rapid turnaround time of one to two min between measurements. However, it lacks the specificity of gas chromato-

graphy, since it cannot distinguish between the proportions and species of different VSC's. In addition, its utility would be improved if accurate laboratory standards were readily available for daily calibration of the machine, as has been used for calibration of organoleptic measurements (Tonzetich and Ng, 1976).

None of the parameters in the present report can be singled out as a reference standard for the measurement of bad breath. As with other odor measurements, subjectivity is to be expected in any organoleptic assay (Doty *et al.*, 1982). Even in pre-calibrated panels of judges, the calibration step itself may introduce bias (Cain, 1989). The results presented here are consistent with those of many other previous reports (Solis-Gaffar *et al.*, 1975; Tonzetich and Ng, 1976; Schmidt *et al.*, 1978; Kostelc *et al.*, 1984) describing relationships between organoleptic measurements and VSC, and reductions of oral malodor after mouthrinse treatment. The patients examined in this study exhibited appreciable levels of intra-oral volatile sulphides, yet the variance of VSC measurements was not well-explained by variations in salivary flow rate, as has been previously suggested (Tonzetich, 1977). Indeed, in the patient population studied herein, the variance in VSC measurements can best be attributed to the cumulative effect of a number of factors, including the amount of supra-gingival dental plaque, the presence of periodontal pockets, gingival inflammation, and age. If these data can be replicated in an appropriately designed clinical study with adequate sample size and a well-described patient population, then the measurement of oral malodor with the portable industrial sulphide monitor may be useful in the diagnosis of bad breath and the screening of patients for periodontal diseases. Although the strategic application of breath monitoring for periodontal diseases has been previously noted with gas chromatography (Tonzetich, 1978; Kostelc *et al.*, 1981, 1984), the use of a simple device such as the sulphide monitor described here appears to be more applicable to chair-side measurement.

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Measurement of Oral Malodor: Current Methods and Future Prospects

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MEASUREMENT OF ORAL MALODOR is complicated by a variety of parameters including complexity of gaseous molecular species, sampling difficulties, temporal variations, choice of suitable subject populations, and lack of agreement on reference standards. Since oral malodor is a perceived olfactory stimulus, direct sampling and assessment by human judges may be the most logical measurement approach. However, as with other psychophysical assessments, human malodor measurement by the human nose may vary widely among and between judges, and consequently cannot be confidently reproduced in other laboratories. Such shortcomings have led several investigators to propose quantitative approaches based on measurement of volatile sulfide compounds which are associated with oral malodor. Highly sensitive and discriminatory measurements of volatile sulfides can be made using gas chromatography although for rapid sampling of larger subject populations, portable sulfide monitors may be more appropriate. Future research in this field should consider: 1) improved and simplified instrumentation for more rapid throughput and reliability; 2) development and definition of reference standards for oral malodor assessment; 3) formulation of clinical studies with appropriately sized, well-defined patient populations; and 4) further development of within mouth, site-specific measurements. *J Periodontol 1992; 63:776–782.*

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Oral malodor (i.e., bad breath, halitosis, *fetor ex ore*) is a common complaint that may periodically affect most of the adult population.[1,2] In the large majority of cases, oral malodor originates in the oral cavity as the result of microbial metabolism.[1,3–5] Malodor of oral etiology may result from microbial metabolism on the tongue dorsum and periodontium, and is modulated by low salivary flow at certain times during the day, food impaction, and diet.[6–12] Non-oral etiologies may include upper and lower respiratory tract conditions, neurologic and gastrointestinal disorders, various systemic diseases, and use of certain drugs.[13]

Oral malodor is a subject of considerable public interest, yet has engendered only limited scientific investigation in part because of the difficulties of measurement. Thus, although a circle of acquaintances might generally agree that a certain person suffers from bad breath, scientific assessment and validation of oral malodor measurements have not been realized. The purpose of this manuscript is to review direct and indirect techniques for measuring malodor, to discuss the problems associated with selecting subjects and sampling, and to raise some prospects for future research in this area.

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Subjective Measurement of Oral Malodor

The most simple and commonly used approach to sample and measure oral malodor is direct nasal sniffing of expelled mouth air. This is often referred to as "organoleptic" or "hedonic" assessment. In a sense, this method may be considered a kind of reference standard of oral malodor measurement. This approach closely simulates the everyday situations in which bad breath is detected. However, such organoleptic measurements also raise several problems.

One concern is that there is considerable variation between judges on the ranking of the unpleasantness of different odors.[14–18] Thus it is not surprising that appraisal of bad breath differs from one judge to another.[18] Furthermore, a wide variety of confounding factors including psychological/physiological states (e.g., hunger, menstrual cycle, head position, degree of attentiveness and expectation[14–16]) influence organoleptic measurements. Moreover, olfactory sampling is best performed by taking a short, rapid sniff,[14] an activity which is difficult to perform repeatedly in exactly the same fashion.

To reduce inter-examiner variations, a panel consisting of several judges is often employed, and the level of malodor is

based on the mean score.[1,7,11,12,18–21] This procedure however, leads to sampling problems. Since it is not feasible for several judges to measure the bad breath of a given subject simultaneously, sequential measurements are often made.[7] This method introduces additional complications; once the oral headspace is sampled for measurement by one judge (e.g., by having the subject breathe out through the mouth), it is unlikely that the concentration and composition of the various gases are reproduced in subsequent samples.

Olfactory assessment of odor is one example of psychophysical measurements that include other sensations such as pain and taste. Such parameters are difficult to quantify, and bad breath is no exception. The intensity of oral malodor observed by human judges is usually graded on a scale consisting of three or more descriptive levels (e.g., no odor, weak, middle, or strong); optimally, the odor judge treats these categories as if separated from one another by equivalent intervals.[18,20,22–24] One of the drawbacks of such scoring is that it is often difficult to decide on intermediate grades (e.g., whether a given sample should be scored "2" or "3" on a scale of zero to 5). Moreover, whereas the zero score usually represents no observable odor, any top score (e.g., extremely foul) is open-ended, and may cover a wide distribution of extremely foul odors. Apparently, no studies have attempted to use continuous scales, which have been introduced in other areas of psychophysics. In early studies of malodor, an osmoscope was used in conjunction with a scale based on the highest dilution at which malodor could be detected.[8,25,26] For reasons that are not clear, linear increases in organoleptic ratings usually correspond to exponential (rather than linear) increments in odorant concentration.[14,24]

Should judges of oral malodor receive prior training or undergo precalibration procedures? The reliability and reproducibility of organoleptic measurements may possibly be improved if the judges are trained to recognize and rate oral malodor. In a recent study comparing an experienced and a novice oral malodor judge, odor scores of the former were more reproducible, and gave higher correlations with instrumental sulfide analysis.[19] Furthermore, agreement among panel judges might be higher if they are previously instructed to assign certain ratings to given levels of odor stimuli.[14–17] However, reproducible "bad breath standards" do not exist, thus a preview of assigned stimuli prior to the experiment [24] is not feasible. Furthermore, training and precalibration procedures might limit individual judgment, thus biasing scoring towards that of the instructor. In one study in which 7 untrained judges independently scored the oral malodor of 75 subjects, inter-examiner correlation coefficients (Spearman) were significant but not high, ranging from 0.14 ($P = 0.14$) to 0.49 ($P < 0.001$). Interestingly, 2 of the judges' ratings appeared to correlate poorly with the other 5 judges, yet correlated with one another to a higher degree.[18] This suggests that certain individuals may respond differently to various components of oral malodor.

While general olfactory tests are available for selecting odor judges,[15,16] little information is available on how judges for bad breath assessment should be selected or excluded. First, since odor perception is purportedly more developed in females than males, it is conceivable that females may be better judges than males.[27] Second, there is a decrease in the ability to perceive odors with increasing age.[27] Third, if people become adapted to their own oral malodor, should potential judges be excluded if their own malodor exceeds a predetermined level? In this context it should be recognized that in the clinical setting, it is the dental professionals who should notice and treat oral malodor. Yet if dentists become habituated to oral malodor just as workers in other foul-smelling surroundings (e.g., tanneries) become accustomed to workplace odors,[14] dentists may have a reduced ability to recognize and assess oral malodor.

Instrumental Analysis of Oral Malodor

Over the past three decades, Tonzetich and coworkers have developed instrumental analysis of oral malodor using gas chromatography (GC) coupled with flame photometric detection.[1,11,22,27–31] These investigations have led to the conclusion that oral malodor is associated primarily with volatile sulphur compounds (VSC). Tonzetich proposed that among the sulphur gases, hydrogen sulfide and methyl mercaptan were the main odor-causing components. Recently, Yaegaki and coworkers have shown that hydrogen sulfide is produced largely from the tongue dorsum, and that malodor from periodontal tissues is enriched with methyl mercaptan and dimethyl disulfide.[12] GC measurement has been used to demonstrate malodor reduction after use of mouthrinses, as well as mechanical oral hygiene techniques.[20–23]

Gas chromatographic measurement of malodor samples has several advantages compared to organoleptic measurement including: 1) separation and quantitative measurement of individual gases; and 2) the ability to measure extremely low concentrations of gases. The main disadvantages of such instrumentation are: 1) relatively high cost; 2) the need for skilled personnel; 3) cumbersome and lack of portability; and 4) the time required for detection and measurement (sample throughput). As a result of the labor-intensive nature of GC sample assessment, many malodor investigations have involved relatively small numbers of subjects. For example, the clinical studies of Tonzetich and coworkers,[11,22,30,31] Solis-Gafar et al.,[32] and Kostelc and coworkers,[6,33,34,37] employed 20, 8, 15, 5, 13, 10, 14, 23, and 17 subjects, respectively (see also Table 1).

There are few published studies on the correlation between organoleptic ratings and the level of VSC as determined by GC techniques. Unfortunately, studies which employed both methods to test the efficacy of various treatments did not provide statistical comparisons of the measurement techniques themselves. In one of the few studies which presented such data, Schmidt et al.[20] compared GC with organoleptic ratings by three judges using a scale of 0 to 3. Thirty-six subjects (18 men and 18 women) who

Table 1: Summary of Some Clinical Oral Malodor Studies

Subject Population (Reference)	Measurement Techniques	Main Findings
5 matched pairs of healthy students, one group abandoning oral hygiene for 30 days (6)	GC-flame photometric analysis of mouth air; GC-mass spectrometric analysis of saliva headspace	Increases in breath and salivary volatiles in experimental group
75 adults (18)	Peak sulphide measurements, using portable monitor, vs. mean of 7 organoleptic judges (both measurements of whole mouth odor)	Significant ($P < 0.001$) correlation ($r = 0.60$) between the 2 methods
41 adults complaining of bad breath (19)	Organoleptic measurements of whole mouth, tongue and floss, sulphide measurements with portable monitor	Steady-state sulfide measurements were most reproducible
102 adult subjects in two separate studies (20)	GC-flame photometric analysis of VSC in mouth air, vs. organoleptic whole mouth measurement by 3 professionally-trained odor judges	Significant correlations between organoleptic results and total hydrogen sulfide + methyl mercaptan
8 male non-smokers with objectionable early morning mouth oder (20)	GC-flame photometric analysis and 8-member organoleptic panel measurement of whole mouth odor	Reductions in sulfides and malodor following oral hygiene procedures
30 adult subjects with over 30 ppb of methyl mercaptan in their early morning mouth air (23)	GC mouth odor analysis; organoleptic analysis by 5 judges through glass tubes, through holes in a partition; microbial sampling for odorigenic microorganisms	Decreases in all measurements following mouthrinsing with active mouthrinse, vs. placebo rinse and water
100 patients with periodontal problems vs. 100 with normal periodontia (26)	Paraffin-stimulated saliva samples with added casein were analyzed for sulphides and indole, following 1 and 3 hours incubation	Saliva from pathological cases putrefied more rapidly than from normal cases, producing higher levels of sulfides and indole.
15 non-smokers, 10–50 years old (30)	GC-flame photometric analysis of VSC in mouth air and putrefied saliva headspace	Suitable system for detecting VSC and measuring treatment effects
5 healthy females 21–30 years old (31)	GC analysis of hydrogen sulphide, methyl mercaptan, and dimethylsulphide in mouth air	Cyclical changes in VSC during menstrual cycle
13 adult males, non-smokers, with good oral health, but high levels of VSC in morning breath (32)	GC-flame photometric analysis of mouth air, of subjects rinsing with either placebo or active mouthrinse	Reductions in hydrogen sulfide (14%) and methyl mercaptan (45%) in the mouthrinse group
7 healthy subjects vs. 7 with periodontitis (33)	GC and GC-mass spectrometry of putrefied saliva headspace; note difference in collection of saliva between the 2 groups	Pyridine and methyl pyridines (picolines) found only in diseased group
13 "normal" vs. 10 with "moderate or advanced periodontal involvement" (34)	GC and GC-mass spectrometry of putrefied saliva headspace	Pyridines and methyl pyridines (picolines) primarily in periodontal group
17 subjects (3 females, 14 males) (37)	GC-mass spectrometry of saliva headspace	Various components of exogenous origin (pollutants, foods, cosmetics) were identified

did not exhibit any overt oral disease other than mild gingival inflammation or dental caries were studied in the first experiment; the second experiment was extended to include 66 subjects (36 males and 30 females). Whereas the correlation coefficients between VSC (hydrogen sulfide + methyl mercaptan) levels and organoleptic scores were low in both experiments (0.28 and 0.35, respectively, using Kendall rank correlation analysis), in the second experiment, the null hypothesis (i.e., no correlation between VSC and organoleptic scores) could be excluded at a higher level of confidence ($P < 0.001$). The extent of variation among the individual judges was not reported.

Recently, the application of an industrial sulfide monitor for measuring gases associated with oral malodor has been reported.[18] In an initial study, highly significant correlations were demonstrated between monitor measurements of 75 subjects, and organoleptic ratings by 7 judges, with an overall Spearman correlation of $r = 0.60$ for log-transformed sulfide scores vs. the mean of the panel of judges.[18] Similar correlations between sulfide concentrations obtained with the monitor and organoleptic ratings of odor were found in subsequent studies.[19,35] Measurements using the monitor have been shown to be more reproducible than organoleptic measurements, and more sensitive to reductions in malodor brought about by mouthrinsing.[19,35] The advantages of the monitor compared to GC include: 1) lower cost; 2) can be operated by non-skilled personnel; 3) portability; and 4) rapid turn-around time between measurements. A further possible advantage is the manner in which the sample is obtained (vide infra). The main disadvantages of the instrument in its present configuration are: 1) its inability to distinguish between individual sulfides; 2) measurement cannot be made in the presence of high levels of ethanol or essential oils, thus precluding assessment of mouthwash efficacy until these components have dissipated; and 3) the instrument may show a slight loss of sensitivity with time, necessitating periodic recalibration.[19] Studies which compare GC and sulfide monitor measurements have not been published to date, but may prove highly instructive. One major difference between the two techniques is the manner of sampling, to be discussed in a later section.

Should Malodor Originating in the Mouth Be Considered a Single Entity?

Both hydrogen sulfide and methyl mercaptan are highly volatile gases which are readily detected by GC. Nevertheless, it is possible that other odorants may be important as well. Whereas the correlations between both VSC and organoleptic measurements show that the two are, at least to

some extent, associated with one another,[18–20] it does not necessarily follow that they are chiefly responsible for oral malodor. This point has been demonstrated in the food industry, in which certain gases have been correlated with poor tasting products yet are not themselves directly responsible for the poor taste.[16] Tonzetich and coworkers sniffed the eluent of the GC and found that hydrogen sulfide and methyl mercaptan were the main malodorous components eluted.[36] However, less volatile components which evade detection might also be involved. Several observations support this view: 1) following use of antiseptic mouthrinses or oral hygiene methods, reductions in both VSC and organoleptic ratings are observed, but the reductions of VSC are much more dramatic than the reductions of organoleptic scores;[11,21–23] 2) bad breath is more noticeable when the mouth dries out (e.g., following fasting,[30] a long period of talking, or sleeping[1,11]), suggesting that malodorous molecules are retained in or beneath the water film covering the oral mucosa but, following drying of the tissue, can be volatilized; 3) despite the possibility that the mixture of oral gases changes the nature of the odor of the individual components, neither hydrogen sulfide nor methyl mercaptan smells like bad breath; and 4) most natural odors owe their characteristic smell to tens or possibly hundreds of volatile component molecules.[14]

To illustrate the potential complexity of the oral gaseous species involved, Kostelc and coworkers found elevated levels of a variety of volatiles in the saliva headspace of subjects with periodontal disease, including dodecanol, tetradecanol, phenol, p-cresol, pyridine, and pyridine, 2-,3-,4-methylpyridine.[33,34] Whereas Tonzetich and coworkers ruled out a possible role for various components based on low volatility, these experiments were based on their release from liquid (saliva) to gas (air), and may not reflect their volatility on dry oral surfaces.

If a number of odorants are involved in malodor of oral etiology, it is worth considering the possibility that their relative levels vary among individuals. For example, there is usually an appreciable difference between odor originating from the tongue and that emanating from dental floss passed interproximally.[19] Yaegaki and Sanada[12] have provided evidence that the hydrogen sulfide/methyl mercaptan ratio in tongue and oral malodor changes as a function of the severity of periodontal disease, although it is unclear whether variations in the concentrations of these two gases can account for the difference in the qualities of odor observed.

Sample Populations

The choice of participants in a clinical study of oral malodor is an important, but largely ignored, aspect of oral malodor research and may be crucial to our understanding of the nature of oral malodor and the homogeneity of the data obtained. For example, the overall contribution of malodor from the tongue dorsum may differ in healthy subjects vs. those with periodontal diseases.[12] One potential approach to improve the homogeneity of the study population would be to include only subjects who think they have bad breath. However, as recently shown, a large proportion of such subjects who are convinced that they suffer from oral malodor do not necessarily present with intense malodor levels.[19]

After deciding upon inclusion criteria, the choice of exclusion criteria may also improve the homogeneity of the data set and thus permit more clear-cut conclusions. For example, because of fluctuations in malodor associated with the menstrual cycle, women may make less suitable subjects than men.[31] Exclusion criteria should also consider smoking, dietary factors (e.g., onions and garlic), medication, time of year, and dental treatment. Exclusion criteria may reduce data variation of the results obtained, but also restrict the findings to the subclasses of subjects examined.

Obtaining the Sample

Bad breath may be compared to other cases of pollution of the ambient atmosphere emanating from confined sources. Points of discharge from confined sources (e.g., smokestacks) have characteristic moisture contents, temperature, shape, discharge rates, dilution, and dispersion variables.[38] Awareness of bad breath comes only after release from the mouth into the open environment. In both cases, the physical conditions at the site of sampling are far different from those at the origin. Thus the method of obtaining the sample itself contributes to bias and variation.

We usually notice bad breath while someone is talking to us. Yet, measurement in such a manner has not been reported. In GC studies, the sample is usually obtained by drawing air from the headspace within the mouth (e.g., with a syringe). Conversely, in subjective organoleptic panel measurements, the subject is instructed to breathe out through the mouth. Both these approaches have drawbacks. In a closed mouth, a concentration gradient probably exists, with the highest levels of odorants proximal to the surfaces from which they emanate. When the mouth is opened for headspace sampling, turbulence may contribute to unrepresentative sampling of mixtures of gases when the syringe sample is drawn and may result in a completely different mix of gases than that obtained by static sampling. In organoleptic measurements, the mouth air is largely diluted by lung air as the subject rapidly breathes out. Neither of these methods replicates the flow of malodor outwards from the mouth during speech, for example. One potential solution to this problem is to blow air into the mouth and measure the effluent emitted from the mouth, having the patient refrain from mouthbreathing during measurement.[26] A similar approach is employed with the portable sulfide monitor, which draws air out of the semi-open oral cavity at a fixed rate while the subject breathes through the nose.[18] Initially, a peak sulfide measurement is observed, which reflects the standing headspace level. Subsequently, as the oral cavity is washed by the continual stream of air sucked into and

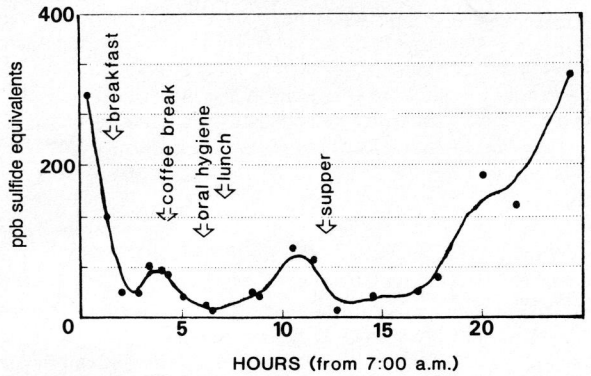

Figure 1. Oral malodor as a function of time of day. Volatile sulfides, in peak ppb hydrogen sulfide equivalents, were measured in a single subject (MR), over the course of a day, starting at 7:00 a.m. Measurements were made using a 1170 portable sulfide monitor as described in reference 19. Significant oral activities are indicated.

from the mouth, the measurement falls to a steady state level. This parameter apparently reflects the contributions of ongoing malodor production, together with mass transfer of odorants from the oral surfaces into the gaseous phase. Indeed, such steady state values proved more reproducible than the initial peak measurements obtained.[19]

Finally, the temporal aspects and frequency of sampling cannot be overlooked. The level of oral malodor in an individual may vary greatly over the course of a single day (Fig. 1). Nevertheless, if subjects are required to refrain from potentially confounding oral activities for several hours prior to examination, reproducible measurements can be made a week apart.[19]

Whole Mouth Versus Site Specific Measurement

It should be emphasized that the measurements described above assess whole mouth malodor, whereas it is likely that the origins of the malodor are localized (e.g., foci on the tongue dorsum, or specific periodontal pockets). Rizzo detected the presence of hydrogen sulfide in individual pockets using paper impregnated with lead acetate.[39] Refinement of such an approach, which would yield quantitative data on malodor production at various oral sites might provide information on metabolism at specific sites in the periodontium and could over the long-term lead to an improved understanding of fluctuations in periodontal disease activity and perhaps other oral diseases (e.g., white lesions of oral mucous membranes, candidiasis). Rosenberg et al.[19] attempted to pinpoint loci of malodor by organoleptic assessment of floss following interproximal passages, as well as gauze rubbed along the tongue. Although there was some statistical association between these parameters and whole mouth assessments, neither of the two techniques proved reliable.

‡Interscan Corp., Chatsworth, CA.

Indirect Measurement of Oral Malodor

The variability of oral malodor measurements and the difficulties in assessing oral malodor directly from the oral cavity have led investigators to employ indirect methods. The approach which has been most widely employed is the measurement of malodor and volatile sulfides in putrefying saliva samples.[1,6,11,12,28–31,36,37,40] In most cases, saliva obtained directly from the mouth possesses little or no foul odor, but after several hours of in vitro incubation, malodor is easily detected, due primarily to breakdown of particulate matter by microorganisms.[12] Whereas some extrapolations between the odor of putrefied saliva and oral malodor may be acceptable, caution should be exercised since the odor of putrefied saliva usually differs appreciably from directly-sampled oral malodor. A second indirect method which has been less frequently employed is the measurement of sulphides and malodor from bacterial isolates and pure cultures.[3–5,23]

Self-Assessment of Oral Malodor

Considering the many hundreds of millions of dollars that are spent annually on mouthwashes, sprays, candies, and chewing gums that are marketed on the basis of breath freshening, it is evident that oral malodor is of concern to a substantial proportion of the North American population. Few individuals seek or receive adequate professional counseling.[41] It has usually been assumed that we do not notice our own oral malodor, presumably because we become adapted (habituated) to it.[1,2,8,38] Thus, people possessing foul bad breath are often unaware of it, whereas many individuals who have relatively low malodor levels may be excessively concerned that they have bad breath. Clearly, self-diagnosis of bad breath is a subject of considerable public interest. There are at least three methods by which people can attempt to diagnose their own oral malodor. Firstly, one may cup one's hands over the mouth and nose, breathe out through the mouth and immediately take a short sniff. In many situations (especially upon arising) malodor may be perceived. Of course, it is difficult to ascertain whether such a self-measurement provides an objective estimation, but nevertheless it may be considered as the minimal level which others might notice. Secondly, patients may be alerted to the foul odor which often accompanies dental floss following interproximal passage. Such odors probably make a significant contribution to whole mouth odor. Finally, one's tongue odor may be conveniently assessed by extending the tongue out as far as possible, licking one's wrist, waiting for several seconds, and then smelling. Although these last two approaches are indirect, they may be of diagnostic and therapeutic value, and are currently under experimental evaluation.

Future Prospects

Oral malodor is a subject of considerable public interest yet, as described above, scientific advances have been stymied by the lack of simplified instrumentation and the need for

detailed molecular studies that dissect the component species of oral gases. Progress in the future will rely on: 1) improved instrumentation with the capacity for multiple species sampling and with high patient throughput; 2) development and general agreement on reference standards of oral malodor gaseous species so that interlaboratory comparisons can be made; 3) design and conduct of clinical studies with appropriate inclusion/exclusion criteria, sample size, and outcome variables; 4) development of site-specific sampling methods within the mouth; and 5) association studies of the relationship between oral malodor and significant oral diseases.

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Day-Long Reduction of Oral Malodor by a Two-Phase Oil:Water Mouthrinse as Compared to Chlorhexidine and Placebo Rinses

Mel Rosenberg, Ilana Gelernter,† Mira Barki,* and Ronit Bar-Ness**

FEW SCIENTIFIC INVESTIGATIONS HAVE ADDRESSED THE ability of mouthrinses to reduce oral malodor for periods longer than 3 hours. In the present report, we have employed simple, recently described techniques to assess the day-long reduction in oral malodor of a novel 2-phase oil:water mouthrinse (TPM), as compared to a corresponding placebo rinse, and to a commercial 0.2% chlorhexidine mouthrinse. Sixty dental students were divided randomly into 3 groups, and instructed to use one of the rinses prior to bedtime and the following morning. Measurements carried out in the late afternoon, about 8 to 10 hours following rinsing, were compared with baseline measurements carried out in the late afternoon of the previous day. Volatile sulphide levels were measured using a portable industrial sulphide monitor. Microbial levels were estimated using a simple rinsing technique employing sterilized milk. These quantitative techniques were corroborated by organoleptic (hedonic) ratings of a single odor judge. Both TPM and chlorhexidine brought about significant decreases in volatile sulphides ($P < 0.05$) as compared to the placebo group. These results were corroborated by the organoleptic data. Similarly, both chlorhexidine and TPM were highly effective in reducing microbial levels as measured by the rinsing technique, in comparison to the placebo group. Chlorhexidine appeared to be more effective than TPM in all measurement categories, although only in the case of microbial activity was there a significant ($P < 0.05$) difference between the two groups. The data showing daylong reduction of malodorous sulphides and microbial levels by TPM extend previous in vitro studies demonstrating its potent microbial desorption properties, and provide the first evidence for its potential clinical efficacy. *J Periodontol 1992;63:39–43.*

Key Words: Halitosis/prevention and control; mouthwashes/therapeutic use; chlorhexidine/therapeutic use.

Mouthrinsing is a common oral hygiene practice dating back to ancient times.[1] Although, in recent years, the plaque and gingivitis-reducing properties of various mouthrinses have been emphasized,[1-3] one of the major concerns which leads to frequent use of mouthrinses is halitosis (bad breath).[2] Bad breath usually originates within the oral cavity itself, due to production of gases (primarily volatile sulfur compounds, VSC) by sequestered deposits of microorganisms, generally under anaerobic conditions.[4,5] Periodontal diseases commonly result in bad breath.[4-6] Other loci of oral microbial putrefaction include the tongue dorsum, areas of food impaction, and improper dental restorations.[4]

Much of the evidence for the efficacy of mouthrinses in reducing bad breath is anecdotal[7] and there are very few publications in the scientific literature on this subject. Long-term malodor reduction has rarely, if ever, been addressed. For example, in the mouthrinse studies of Pitts et al.,[8] Schmidt and Tarbet,[9] and Tonzetich,[10] data on malodor reduction were given for up to 3 hours following use.

We have recently shown that 2-phase, oil:water formulations containing low concentrations of cationic surfactants such as cetylpyridinium chloride efficiently bind[11] and desorb[12,13] oral microorganisms. In the present study, we have compared the long-term effect of one such 2-phase

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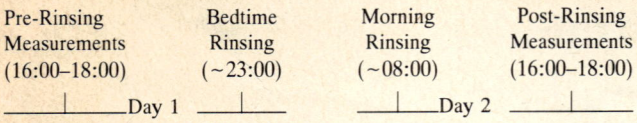

Figure 1. Summary of experimental design.

mouthwash (TPM)[‡] formulation in reducing malodor and microbial levels with a placebo rinse[‡] and an antiseptic mouthrinse containing 0.2% chlorhexidine gluconate (Corsodyl) (CHM).[§] A portable, industrial sulphide monitor[14] was employed to measure volatile sulphides; these measurements were corroborated by organoleptic (hedonic) measurements by a single judge. The effect of the mouthrinses on microbial levels was assessed using the Oratest.[‖] [15,16] The results demonstrate that mouthrinsing with appropriate formulations can result in day-long reduction in bad breath-associated parameters.

MATERIALS AND METHODS

Experimental Design

The experimental design of the present study is summarized in Figure 1. The study took place on March 15 and 16, 1989 at Tel-Aviv University. Sixty dental student volunteers were randomly divided into 3 groups: one group (n = 22) received TPM;[‖] the second (n = 19) placebo rinse,[‖] and the third (n = 19) CHM.[§] No exclusion criteria were employed. The 2-phase mouthwash formulation consisted of an aqueous phase containing cetylpyridinium chloride (0.05%), food color which also serves as a biological stain (FD&C blue No. 1),[16] and artificial sweetener, overlaid with a discrete oil phase containing a mixture of olive oil and essential oils. The placebo rinse contained the same concentrations of food color and sweetener, but without the CPC and oil phase. All mouthrinses were provided in standard brown glass bottles. Volunteers rinsed for two consecutive 30-second periods, directly before bedtime on day 1, and again early the next morning of day 2. Volunteers were instructed to shake the bottles prior to use, and to refrain from rinsing with water, eating, or drinking for at least 30 minutes following rinsing. No other changes regarding oral hygiene were instructed. Pre-rinsing measurements were performed in the late afternoon of day 1. Post-rinsing measurements were performed in the late afternoon of day 2; i.e., about 8 to 10 hours following the previous morning rinse.

Measurements

Quantitative measurements of volatile sulphides were carried out using the Interscan 1170 monitor,[¶] 1 ppm full-scale, connected to a pen recorder. Measurement was carried out essentially as previously reported,[14] except that disposable straws, rather than Teflon tubing, were employed. This instrument is based on an enclosed liquid electrochemical cell, through which the gas sample passes at a constant flow rate, and is sensitive to both hydrogen sulphide and mercaptans (M. Shaw, personal communication Oct 28, 1991.). Volunteers were asked to refrain from talking for 5 minutes prior to measurement. The monitor was zeroed on ambient air, and measurement performed by inserting a disposable 1/4″ plastic straw approximately 4 cm into the partially opened oral cavity. The volunteer was asked to breathe through his/her nose during measurement. Results were recorded as peak ppb sulphide equivalents.

Organoleptic estimations were carried out by a single judge, following monitor measurements, as previously described.[14] For organoleptic rating, volunteers were instructed to exhale briefly through the mouth, at a distance of approximately 10 cm from the nose of the judge. Organoleptic results were rated on a scale of 0 to 5 as follows: 0 = no appreciable odor; 1 = barely noticeable odor; 2 = slight, but clearly noticeable odor; 3 = moderate odor; 4 = strong odor; and 5 = extremely foul odor. All measurements were conducted in a blind fashion.

Microbial levels were estimated using the Oratest, a technique which measures the rate of oxygen depletion in expectorated milk samples. The technique has been previously described and shown to correlate with microbial counts and clinical parameters.[15,16] Briefly, volunteers rinsed for 30 seconds with 10 ml of sterile milk. Following expectoration, 3 ml of the sample were added to test tubes containing 0.12 ml of methylene blue solution (0.1%), and the time required for a blue-to-white color change over a 6 mm diameter at the bottom of the test tube was recorded. The test was performed directly following the volatile sulphide measurements.

Statistical Analyses

One way analysis of variance (ANOVA) was used to determine significant effects among the three groups. Pairwise comparisons between groups were made using the Scheffe procedure, at the $P = 0.05$ significance level. Spearman correlation coefficients were employed to test for correlations among the results of the different measurement techniques.

RESULTS

Comparisons of the effects of oral rinsing with the placebo mouthwash, TPM, and CHM, as measured by the sulphide monitor, the Oratest, and organoleptic assessment, are shown in Tables 1 through 3, respectively. Initially, the CHM group gave slightly higher sulphide and organoleptic scores, as well as shorter Oratest times; these baseline differences, however, were not significant (ANOVA). Similarly, there were no significant differences among the three groups in the number of hours which had passed since last eating or drinking, prior to the tests. The results of the sulphide mea-

[‡]AGIS, Ltd. Tel-Aviv, Israel.
[§]Corsodyl, ICI, Imperial Chemical Industry, PLC, Macclesfield, UK.
[‖]Tiberias Medical Center, Tiberias, Israel.
[¶]Interscan Corp, Chatsworth, CA.

Table 1. Sulphide Monitor Measurements*

	Placebo Rinse (n = 19)	TPM (n = 22)	CHM (n = 19)	*P*(ANOVA)
Before rinsing	1.691 ± 0.372	1.664 ± 0.232	1.717 ± 0.306	0.8595
Change	0.041 ± 0.316	−0.205 ± 0.225*	−0.308 ± 0.292†	0.0009

*Results are presented as mean log ppb sulphide equivalents ± standard deviation.
†Significantly different from placebo at $P < 0.05$ (Scheffe procedure).

Table 2. Oratest Measurements*

	Placebo Rinse (n = 19)	TPM (n = 22)	CHM (n = 19)	*P*(ANOVA)
Before rinsing	1.936 ± 0.499	1.943 ± 0.420	1.708 ± 0.251	0.1166
Change	−0.218 ± 0.367	0.456 ± 0.572†	1.009 ± 0.489‡	0.0001

*Results are expressed as mean log minutes required for color change ± standard deviation.
†Significantly different from placebo at $P < 0.05$ (Scheffe procedure).
‡Significantly different from both placebo and TPM at $P < 0.05$ (Scheffe procedure).

Table 3. Organoleptic Scores*

	Placebo Rinse (n = 19)	TPM (n = 22)	CHM (n = 19)	*P*(ANOVA)
Before rinsing	1.316 ± 1.293	1.500 ± 1.058	1.737 ± 1.240	0.5556
Change	0.053 ± 1.129	−0.500 ± 1.535	−1.316 ± 1.250†	0.0091

*Scores were recorded on a scale of 0 to 5 as described in Materials and Methods. Results are presented as mean scores ± standard deviation.
†Significantly different from placebo at $P < 0.05$ (Scheffe procedure).

Table 4. Correlations of Baseline Measurements Among the Techniques Employed*

	Oratest	Organoleptic Rating
Sulphide measurement	r = −.0977 *P* = 0.229	0.5954 *P* < 0.001
Oratest		−0.1164 *P* = 0.188

*Spearman correlations among the baseline data are presented for all 60 participants. The levels of significance are presented in italics.

Table 5. Correlations Between Individual Changes (post-rinse vs. pre-rinse) Among the Techniques Employed*

	Oratest	Organoleptic Rating
Sulphide measurement	r = −0.5449 *P* < 0.001	0.4238 *P* < 0.001
Oratest		−0.3541 *P* = 0.003

*Spearman correlations among the individual changes (post-rinse vs. pre-rinse) among the three techniques are presented for all 60 participants. The levels of significance are presented in italics.

surements and Oratest were transformed into base 10 logarithmic values to obtain a normal distribution.[14-16]

Comparison of the three groups, based on sulphide measurements, is summarized in Table 1. The overall decrease in sulphide levels following rinsing was statistically significant in both TPM and CHM groups as compared to the placebo group. No significant difference was found in the reductions in the TPM versus CHM groups.

Table 2 shows the comparison among the three groups, based on Oratest scores. Higher Oratest values indicate lower microbial levels,[15] and correspond to lower plaque and gingival scores.[16] No differences were found among the groups in the pre-rinsing scores (ANOVA). However, the overall changes in Oratest scores of both TPM and CHM groups were significantly increased with respect to the placebo group. The increase in Oratest scores was significantly greater in the CHM, as opposed to TPM, group.

Comparison of the organoleptic assessments of the three groups is presented in Table 3. Large variations in scores were obtained in all groups. Nevertheless, the decrease in scores in the CHM group was significant with respect to the placebo (but not TPM) group.

Correlations among the measurement techniques themselves are summarized in Tables 4 and 5. Correlations among the baseline (pre-rinse) measurements are shown in Table 4. Correlations among the individual changes (post-rinsing vs. pre-rinsing) recorded by the various measurement techniques are provided in Table 5. As expected, sulphide monitor scores and organoleptic ratings provided highly significant correlations, both in baseline results (Table 4), as well as in the individual changes recorded by the two techniques (Table 5). Baseline correlations between Oratest scores and the other two measurements were not significant (Table 4). However, as shown in Table 5, the changes recorded by

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the Oratest correlated significantly with those obtained using the sulphide monitor ($r = 0.5490$, $P < 0.001$) and organoleptic assessment ($r = 0.3302$, $P = 0.005$).

DISCUSSION

In the present manuscript, we have assessed day-long reductions in malodor-associated variables, following rinsing with two active mouthwashes, as opposed to a placebo rinse. Sixty volunteers, randomly divided into three groups, rinsed before bedtime and the following morning with one of the mouthwashes. Measurements carried out some 8 to 10 hours later (i.e., in the late afternoon) were compared with baseline measurements carried out at about the same time on the previous day. Measurements were carried out in a blind fashion using two recently developed techniques, measurement of oral sulphides using a portable monitor,[14] and measurement of oral microbial levels, based on the rate of oxygen consumption in expectorated milk samples.[15,16] These measurements were corroborated by organoleptic (hedonic) ratings of a single odor judge (RB) who participated in previous investigations.[14]

The two-phase oil:water mouthrinse (TPM) formulation employed in the present study is based on previous research showing that: 1) a high proportion of oral microorganisms adhere to oil droplets;[17] 2) this adhesion can be further enhanced by appropriate concentrations of cetylpyridinium chloride;[11] and 3) in a comparison of desorption of a model bacterial film by the formulation, as compared to 10 commercial mouthrinses, TPM formulations proved highly effective.[12] Furthermore, TPM formulations reduced malodor levels in pilot studies (data not shown). In the present study, the 2-phase mouthwash was compared with a placebo rinse, containing the same color and sweetening agent as TPM, but without the active agents; i.e., cetylpyridinium chloride and the oil phase. As a positive control, Corsodyl was selected, because of previous reports of malodor reduction by chlorhexidine-containing sprays[7] and dentifrices[18] and the excellent substantive antibacterial properties reported for chlorhexidine formulations.[19]

The results showed that TPM significantly reduced oral sulphides, as compared with the placebo rinse. CHM appeared to be somewhat more effective than TPM in reducing sulphide levels, although the two groups were not significantly different following the rinsing regimen. The reduction in sulphides was corroborated by the decrease in organoleptic scores, which was significant in the case of CHM. The effect of CHM in reducing organoleptic ratings appeared to be more pronounced than that of TPM, although the difference between the two groups was not significant.

Both mouthrinses brought about highly significant, dramatic decreases in the level of oral microorganisms, as determined by the Oratest. Initial studies have shown that this test is extremely sensitive to reductions in oral microbiota following mouthrinsing.[20] Indeed, the drastic decreases brought about by the chlorhexidine rinse were significantly different than both TPM and placebo results. Similar reductions in levels of aerobic oral microbiota up to 5 hours following rinsing with a 0.12% chlorhexidine mouthrinse were recently demonstrated by Kayrouz et al.[19] However, in an in vitro desorption experiment, Corsodyl removed only 15% of the microbial film, as opposed to 98% for TPM.[12] These data, taken together, suggest that the dramatic reductions in malodor observed with the chlorhexidine are due primarily to its substantive, long-term antibacterial effect, and relatively high concentration (0.2%), rather than its microbial desorption ability.

Of additional interest are the correlations between the various measurement techniques employed (Tables 4 and 5). We have recently demonstrated that the results obtained with the sulphide monitor are highly significantly related to the average scores of an organoleptic panel of seven judges.[12] Similar correlations were found here between sulphide levels and organoleptic ratings, both in baseline measurements, as well as in the changes measured by either technique. Recently, in a study of 41 subjects complaining of bad breath, among the malodor and periodontal-related tests employed, sulphide monitor results were the most reproducible.[21] These data, taken together, support the use of sulphide monitors in chairside measurement, and clinical investigation.

The correlation of Oratest scores to the results of the other two techniques appears to be more complex. Whereas baseline correlations provided non-significant correlations between Oratest and both organoleptic as well as sulphide scores (Table 4), the individual changes measured by the Oratest provided highly significant correlations to the changes in both organoleptic and sulphide scores (Table 5). Previous studies have shown that the Oratest yields highly significant correlations with oral microbial counts,[15] as well as plaque and gingival index;[16] plaque index was significantly correlated with malodor in a 1988 study.[2] Moreover, in an initial study encompassing a population of 40 patients complaining of bad breath, modest but significant correlations were observed between Oratest and sulphide monitor levels ($r = -0.454$; $P < 0.01$) as well as with organoleptic scores ($r = 0.459$; $P < 0.01$) (Rosenberg and Barki, unpublished data). Finally, since both malodor and the Oratest appear to be related to oxygen depletion in the oral cavity,[6,15] a correlation between the two parameters might be anticipated. One possibility for the poor baseline correlation found here may be the population studied; i.e., a relatively homogeneous, non-symptomatic group of dental students. Indeed, one of the problems associated with clinical malodor studies is subject populations.[4] The above notwithstanding, the changes measured by the Oratest appear to be highly correlated with changes measured by the other two techniques (Table 5). Thus, while the extent to which the Oratest measures a function which is physiologically related to sulphide or organoleptic scores is unclear from the data presented here, the changes in scores of all three techniques, appear to be related (Table 5).

In summary, the data suggest that both the mouthrinses tested brought about day-long, statistically significant reductions in volatile sulphide and microbial levels, as compared to the placebo rinse control. Both mouthrinses brought about corresponding decreases in organoleptic scores, but only in the case of CHM was the decrease significant. Whereas only in the Oratest category were the results of CHM significantly different from those of TPM, the chlorhexidine rinse appeared more effective in all techniques tested. To our knowledge, the data presented are the first demonstrating day-long reduction of malodor by mouthrinses. Chlorhexidine rinses have several undesirable side effects (discoloration, changes in taste perception, occasional irritation, and desquamation) and are usually not advised for continuous, long-term usage.[1] However, a short-term chlorhexidine rinsing regimen may be of potential diagnostic benefit in helping to determine whether the presenting malodor is of an oral etiology.[21]

Two-phase oil:water combinations such as that studied here have several possible advantages over single-phase mouthwashes: 1) their relative effectiveness in binding and desorbing oral microorganisms;[11,12] 2) since the bacteria and oral debris are stained blue by the food color, they can be macroscopically and microscopically observed to adhere to the oil droplets following rinsing, affording direct observation and self-assessment;[13] and 3) the TPM formulations do not contain alcohol, a substance which has potentially adverse affects; e.g., dehydration, addiction, and irritation of oral and gastrointestinal mucosa. The present study provides the first clinical data supporting the potential use of TPM formulations for reduction of malodor and microbial levels. Further studies on the clinical effects of 2-phase oil:water formulations are in progress.

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Initial Testing of a Novel Urine Culture Device

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The Diaslide urine culture device consists of a hinged case containing two opposing agar media separated by a sampler with a handle at one end and two bent sampler tips at the opposite end. The tips of the sampler are first dipped into the urine. The sampler is then pulled out through the casing, simultaneously inoculating both agar surfaces with a streaking dilution. As a result, individual colonies can be observed even when bacterial concentrations exceed 10^6 CFU/ml. The number of colonies on the Diaslide correlated linearly with CFU per milliliter as determined by dilution plating. The clinical performance of the Diaslide was compared with those of ordinary dipslides and conventional cultures with a sample of 473 prescreened hospital urine specimens. The sensitivity, specificity, and positive predictive value of Diaslide versus those of culture at the 10^4-CFU/ml cutoff level were 97.5, 98.3, and 98.3%, respectively, compared with 98.8, 95.7, and 97.2%, respectively, for dipslide versus culture. Similar results were found at the 10^5-CFU/ml cutoff level. Only 5.5% of the Diaslides required subculturing, compared with 14.7 and 9.4% of the dipslides and conventional cultures, respectively. The Diaslide proved more convenient than an ordinary dipslide for sampling low volumes of urine. These data suggest that the Diaslide is a simple, effective device for culturing of urine specimens.

Urine is one of the few biological specimens submitted to the clinical laboratory that require analysis of both the microbial species and the microbial concentration (9). Quantitative, conventional culture often employs a streaking dilution of the urine sample on solid media with a calibrated loop. This method enables appraisal of the microbial concentration in the sample and a presumptive initial identification, and it provides individual colonies for further evaluation (e.g., antibiotic susceptibility studies). One major disadvantage of this approach is that the urine sample itself must be transported to the microbiology laboratory, where it is then plated out by skilled personnel. Since urine is an excellent medium for bacterial culture (1), the number of bacteria present may increase rapidly in the interim. Once the urine is applied to a solid (e.g., agar) surface, however,

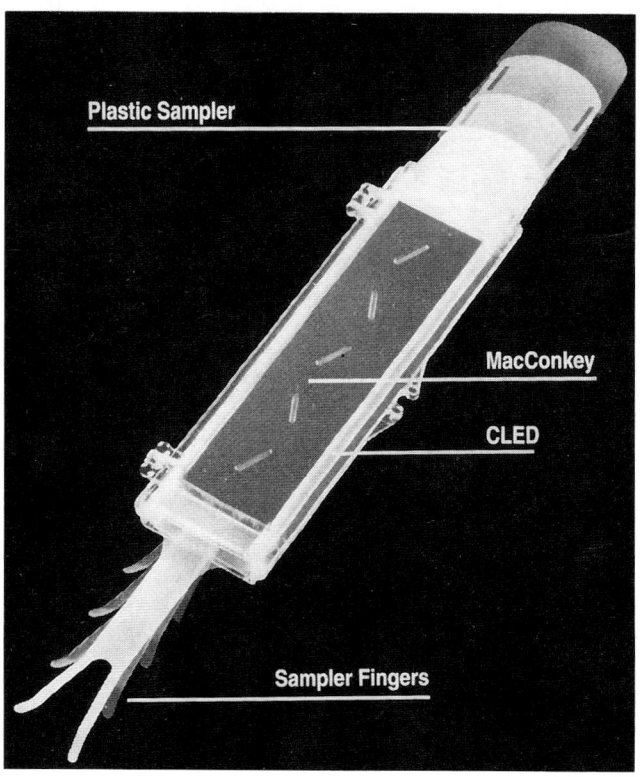

FIG. 1. Design of the Diaslide.

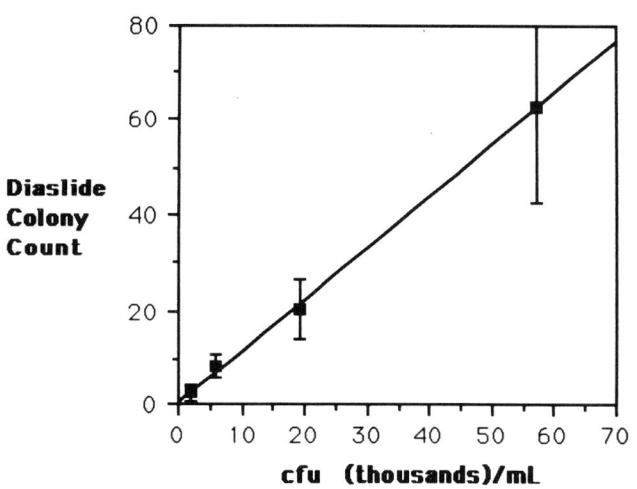

FIG. 2. Correlation between numbers of colonies on the Diaslide and CFU per milliliter in seeded urine samples. Urine, seeded with different numbers of *E. coli* cells, was sampled by using Diaslides (n = 20 for each bacterial concentration). The average Diaslide counts ± standard deviation (CLED) are plotted as a function of CFU per milliliter. Similar correlations (not shown) between enumeration of colonies on the MacConkey surface of the Diaslide and that by dilution plating were found.

* Corresponding author.

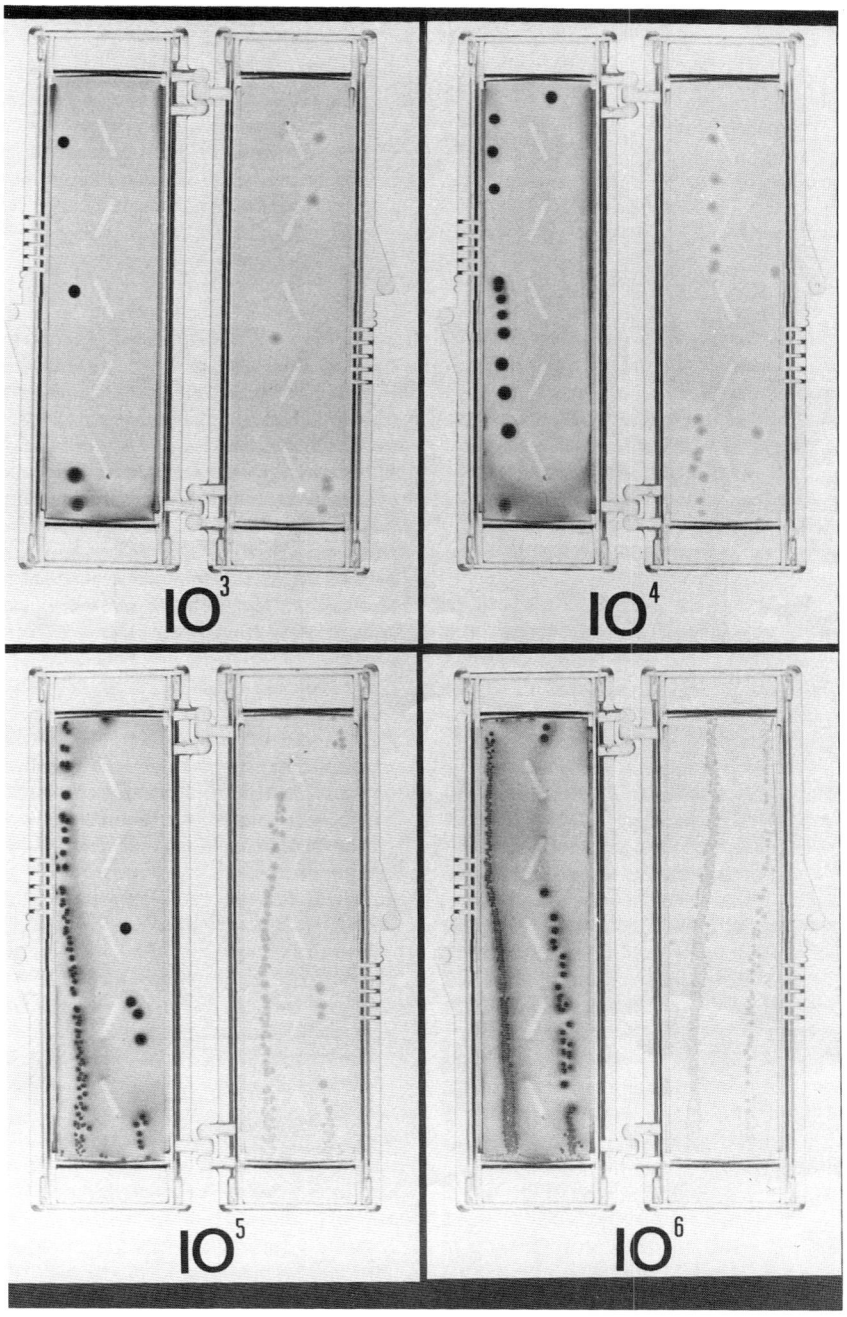

FIG. 3. Diaslide reference chart showing representative results ranging from 10^3 to 10^6 CFU/ml.

each multiplying bacterium yields only a single colony on subsequent incubation.

The requirement for a simple method of applying urine to solid surfaces at the collection site has yielded a variety of approaches, including filter paper strips (12), agar-coated pipettes (10), spoons (13, 14), glass slides (4), and, ultimately, plastic commercial "dipslides." The urine dipslide, with agar medium on each side of an immersible plastic paddle, usually employs a nonselective, electrolyte-deficient medium enriched with cystine (CLED) (13) and a medium selective for gram-negative bacteria (MacConkey or eosin-methylene blue). The dipslide has been shown previously to be a cost-effective, simple device for the detection of bacteriuria in both inpatient and outpatient settings, providing results comparable to those obtained by standard plate-streaking methods (5–8, 11, 15, 16, 18–20).

The conventional urine dipslide has several disadvantages which compromise its usefulness: (i) at concentrations in urine of $\geq 10^6$ CFU/ml, confluent growth is often obtained, complicating detection and subculturing, (ii) sampling of low volumes of urine with the dipslide is cumbersome, (iii) since a large amount of urine is absorbed by the immersed agar surfaces, there is a possibility of false-negative results due to carryover of inhibitory agents present in the urine, and (iv) the condensation which forms on the outer vial during incubation hinders examination and necessitates unscrewing

TABLE 1. Comparison of Diaslide and conventional culture

Cutoff (CFU/ml) and result by Diaslide[a]	No. of the following result by conventional culture[b]			Total
	Positive	Negative	Doubtful	
10^{4}[c]				
Positive	238	4	12	254
Negative	4	157	43	204
Doubtful	1	3	11	15
Total	243	164	66	473
10^{5}[d]				
Positive	206	5	3	214
Negative	4	226	5	235
Doubtful	2	5	17	24
Total	212	236	25	473

[a] Based on comparison with those of the reference chart in Fig. 2.
[b] Based on enumeration of colonies on plates.
[c] At a cutoff of 10^4, culture and Diaslide positives are those judged as $\geq 10^4$ CFU/ml; those judged as $\leq 10^3$ CFU/ml are considered negative, and those between 10^3 and 10^4 CFU/ml are recorded as doubtful.
[d] At a cutoff of 10^5, culture and Diaslide positives are those judged as $\geq 10^5$ CFU/ml; those judged as $\leq 10^4$ are considered negative, and those between 10^4 and 10^5 are recorded as doubtful.

of the cap and removal of the attached slide. Since the majority of urine cultures are negative, this compromises the device's user-friendliness and lengthens the processing time.

The purpose of the present investigation was to develop and test a novel device (17) which combines the advantages of both conventional culture and dipslides. Its effectiveness was evaluated by using reconstituted urine specimens and tested in a clinical trial to compare it with the effectiveness of conventional dipslides and culturing.

TABLE 2. Comparison of Diaslide with dipslide

Cutoff (CFU/ml) and result by Diaslide[a]	No. of the following result by dipslide[b]			Total
	Positive	Negative	Doubtful	
10^{4}[c]				
Positive	250	2	2	254
Negative	5	191	8	204
Doubtful	3	1	11	15
Total	258	194	21	473
10^{5}[d]				
Positive	213	1	0	214
Negative	6	229	0	235
Doubtful	3	7	14	24
Total	222	237	14	473

[a] Based on comparison with those in the reference chart in Fig. 2.
[b] Based on comparison with those in the manufacturer's reference chart.
[c] At a cutoff of 10^4, dipslide and Diaslide positives are those judged as $\geq 10^4$ CFU/ml; those judged as $\leq 10^3$ CFU/ml are considered negative, and those between 10^3 and 10^4 CFU/ml are recorded as doubtful.
[d] At a cutoff of 10^5, dipslide and Diaslide positives are those judged as $\geq 10^5$ CFU/ml; those judged as $\leq 10^4$ are considered negative, and those between 10^4 and 10^5 are recorded as doubtful.

TABLE 3. Summary of statistical data[a]

Cutoff (CFU/ml) and technique	Result (%) compared with that by Diaslide				
	Sensitivity	Specificity	Positive predictive value	Negative predictive value	Total agreement[b]
10^{4}					
Culture	98.3	97.5	98.3	97.5	86
Dipslide	97.8	99.6	99.2	97.4	95.6
10^{5}					
Culture	98.1	97.9	98.1	97.8	94.4
Dipslide	97.3	99.6	97.3	99.6	96.4

[a] Sensitivities, specificities, positive predictive values, and negative predictive values were calculated exclusive of doubtful results.
[b] Total percent agreement was calculated as (number of true positives + number of true negatives + number of true doubtfuls) · 100/total.

MATERIALS AND METHODS

Description of the Diaslide. A schematic diagram of the Diaslide and its component parts is shown in Fig. 1. The casing consists of two hinged plastic sections, each containing agar medium (CLED and MacConkey) and folded with the two agar surfaces facing each other. A specially designed plastic inoculator (sampler) lies between the agar surfaces. At the V-shaped sampling end of the inoculator are two bent tips ("fingers") which are dipped into the urine sample. The sampler is then pulled out through the device, effecting simultaneously the inoculation and dilution streaking of both agars. Each agar surface is thus streaked by the tip of one of the tips, as well as by the bent "joint" of the other tip. The tip inoculation yields a streaking dilution of several orders of magnitude, whereas inoculation by the joint yields a relatively uniform spreading of the sample. Following inoculation, the sampler is discarded and the case is placed in a specially designed tray for upright incubation. Subsequent growth can be observed directly without opening the device. For subculturing of positive samples, the hinged casing is opened.

Microbial strains and growth conditions. *Escherichia coli* ATCC 25922 was maintained on brain heart infusion plates and inoculated into brain heart infusion broth (Difco Laboratories, Detroit, Mich.). Following overnight growth with shaking at 30°C, bacterial suspensions were frozen at −70°C in 3-ml aliquots. Prior to the experiments, aliquots were allowed to equilibrate to room temperature and serial dilutions were performed with fresh sterile urine to yield final concentrations between approximately 10^3 and 10^7 CFU/ml. For viable counts, 10-μl aliquots (at least 10 samples for each concentration) of the appropriate seeded urine samples were applied to brain heart infusion agar plates, and colonies were enumerated. Dipslides (Diagnostic Pasteur, Mames-la-Coquette, France) were inoculated by being immersed in seeded urine samples. Diaslides were inoculated by dipping the tips of the samplers approximately 2 cm into the urine samples and then drawing each sampler out through the casing.

In order to test the effect of an antibiotic on results, fresh urine was seeded with *E. coli* to a concentration of approximately 10^5 CFU/ml and ampicillin (Sigma, St. Louis, Mo.) was added to a concentration of 2 mg/ml. Dipslides (Pasteur) and Diaslides were inoculated with this suspension, and the results were compared with results for control urine containing the same bacterial concentration in the absence of an antibiotic.

Clinical experiments. An initial clinical evaluation was

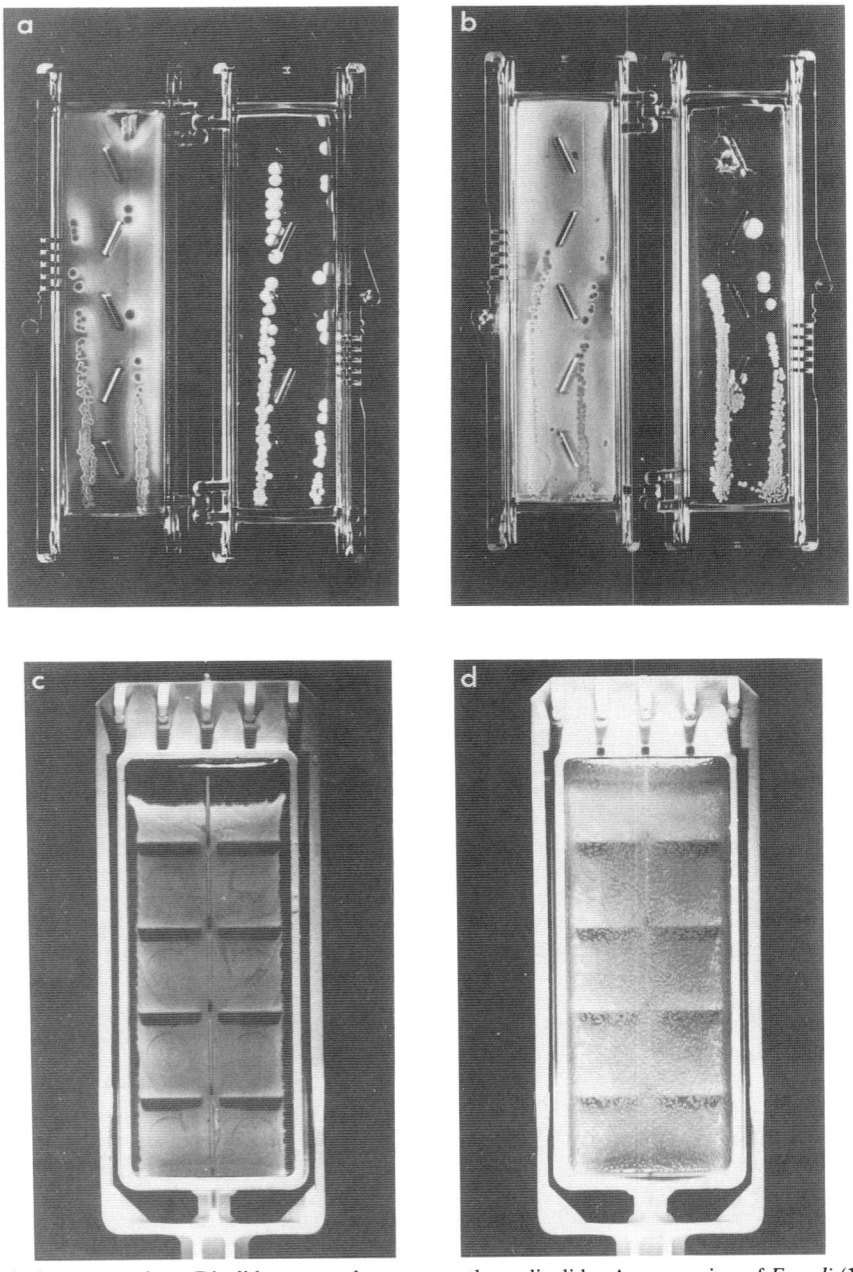

FIG. 4. Effect of antibiotic on growth on Diaslide versus that on growth on dipslide. A suspension of *E. coli* (10^5 CFU/ml) gave normal results on Diaslides whether or not ampicillin was present (a and b, respectively). However, no growth on the dipslide which was immersed in antibiotic-containing urine (c) was observed, compared with that on the control dipslide, which was dipped into the same suspension without added antibiotic (d).

carried out with urine samples which were obtained at random from 700 patients. Thirty percent of the samples were from geriatric and chronically ill hospitalized patients, and 70% of the samples were from other hospital wards and outpatient clinics. Urine samples were prescreened for catalase activity (Uriscreen; Diatech Diagnostica, Ltd., Rehovot, Israel) (3) in order to increase the proportion of positive cultures tested. Catalase-positive urine samples (n = 473) were then evaluated by three techniques: Diaslide, standard dipslide, and conventional culture. Diaslides (Diatech Diagnostica Ltd.) and dipslides (Uricult; Orion Diagnostica, Espoo, Finland) were used and interpreted according to the manufacturer's instructions and reference charts. Conventional culture was carried out with MacConkey and blood agar plates prepared with BBL culture media (Eldantech, Jerusalem, Israel) by using 10-μl disposable loops (Quad-Loops; Miniplast Ein Shemer, Kibbutz Ein Shemer, Israel). Cultures were incubated at 37°C in ambient air for 24 h.

Microbial identification and enumeration were performed with colonies isolated from agar plates. Identification of colonies was carried out by standard methods (2). In cases of mixed growth, the two major colony types were identified to the species level and the combined number of CFU per milliliter was used for the analyses.

RESULTS AND DISCUSSION

In order to compare colony numbers on a Diaslide with the cell density of a sample, urine was seeded with *E. coli* cells at concentrations which enabled enumeration of the number of colonies on the Diaslide. A linear correlation between Diaslide counts and plate counts at levels above 10^3 CFU/ml was observed (Fig. 2). As with ordinary dipslides, semiquantitative estimations of microbial levels could be carried out by comparison with those of reference Diaslides (Fig. 3).

An initial clinical evaluation in which Diaslide results were compared with those of conventional dipslides and plate cultures was conducted. Among the 473 cultures assayed, 243 (51%) were positive at the $\geq 10^4$ level and 212 (45%) were positive at the $\geq 10^5$ level (conventional culture). *E. coli* strains (n = 108), coagulase-negative staphylococci (n = 113), *Proteus* spp. (n = 83), *Klebsiella* spp. (n = 59), *Pseudomonas* spp. (n = 52), *Enterococcus faecalis* (n = 50), *Candida* spp. (n = 36), diphtheroids (n = 33), lactobacilli (n = 16), *Acinetobacter* spp. (n = 15), coagulase-positive staphylococci (n = 12), *Serratia* spp. (n = 7), *Enterobacter* spp. (n = 6), *Citrobacter* spp. (n = 4), beta-hemolytic streptococci (n = 4), and *Salmonella* spp. (n = 1) were among the 599 strains isolated from the positive Diaslides. The ability of the catalase prescreening test to identify *Enterococcus* infections was attributed to the presence of leukocytes in such specimens (3).

Results of the Diaslide versus those of standard plate cultures for cutoffs of 10^4 and 10^5 CFU/ml are shown in Table 1. Results of the Diaslide versus those of conventional dipslides are shown in Table 2. Table 3 presents a comparison of the sensitivities, specificities, positive predictive values, and total agreement of the various techniques. With the exception of a single category (86% percent total agreement between Diaslide and culture at the 10^4 cutoff, mainly due to 43 doubtful results for culture which scored as Diaslide negatives), Diaslide gave results comparable to those of both conventional culture and the dipslide technique.

In the present study, prescreened urine samples were employed in order to increase the proportion of positive cultures. This approach appears valid, since negative cultures were correctly identified with a high rate of success by using the Diaslide, with specificities ranging from 97.5 to 99.6% (Table 3). Nevertheless, future studies using nonscreened urine samples should also be performed.

Several potential advantages of the Diaslide became evident during the clinical evaluation. (i) Isolation of individual colonies was superior with the Diaslide, as only 5.5% of the Diaslides required subculturing, compared with 14.7 and 9.4% for the dipslide and plating techniques, respectively ($P < 0.001$ and $P < 0.03$, respectively, by the McNemar test). (ii) The Diaslide was much more convenient than conventional dipslides for sampling low volumes of urine (e.g., ca. 2 to 10 ml). (iii) Whereas dipslides had to be unscrewed following incubation for growth to be observed (because of condensation on the walls of the casing), microbial growth on Diaslide samples could be determined without removing the latter from the incubation stand. (iv) In general, growth on Diaslides was much more easily recognized than growth on dipslides because of the contrast between the isolation tracks and the surrounding medium, readily visible through the transparent casing. This was most evident with high concentrations of nonfermentative microorganisms which form transparent confluent films on conventional dipslides but are readily observed on Diaslides. (v) Since growth on the Diaslide is concentrated along two well-defined streaking lines, spurious exogenous contaminants were easy to distinguish.

One additional potential advantage of the Diaslide is the elimination of false negatives due to carryover of antibacterial agents from the urine (e.g., antibiotics) onto the agar surface. In order to illustrate this point, urine seeded with bacteria in the presence of ampicillin was sampled with the Diaslide and a dipslide (Fig. 4). Whereas the presence of ampicillin barely affected growth on the Diaslide, microbial growth on the dipslide was completely inhibited. Further experiments are under way to test the potential clinical relevance of this observation.

These data suggest that the Diaslide is a simple-to-use microbial-sampling device which incorporates the main advantages of conventional cultures and dipslides without compromising clinical performance. Whereas the Diaslide is currently being developed for urine testing, experiments are under way to examine its ability to sample other liquids, feces, and foods.

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Isolation of a *Candida albicans* DNA Sequence Conferring Adhesion and Aggregation on *Saccharomyces cerevisiae*

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***Candida albicans* is an opportunistic pathogen which may give rise to superficial and systemic infections. In the present study, *C. albicans* adhesion was studied by expression of *C. albicans* DNA sequences encoding adhesion functions in a nonadherent strain of *Saccharomyces cerevisiae*. Adherent transformant cells of *S. cerevisiae* harbouring a *C. albicans* genomic library cloned in a yeast-*Escherichia coli* shuttle vector were selected by using tissue culture-treated polystyrene as the attachment substratum. One transformant exhibited enhanced adhesion to treated and untreated polystyrene as well as autoaggregation, unlike control cells bearing the vector alone. Analysis of this clone revealed an insert of ca. 4.5 kb from *C. albicans*. Curing of the plasmid resulted in loss of adhesion and autoaggregation properties. A subclone bearing a reduced insert of 3.3 kb retained the ability to autoaggregate, to bind to treated and untreated polystyrene, and to adhere to buccal epithelial cells, unlike appropriate controls. Further subcloning of the insert to 2.7- and 1.9-kb fragments resulted in incremental decreases in adhesion and autoaggregation, whereas smaller fragments did not confer these properties. Hybridization of the 2.7-kb segment with *C. albicans* and *S. cerevisiae* DNA confirmed its origin as a single-copy sequence in the *C. albicans* genome as well as the absence of a homologous sequence in the genome of *S. cerevisiae*. The data suggest that the adhesion and aggregation phenomena of the transformant cells are related to expression of a *C. albicans* surface antigen encoded by the cloned DNA fragment.**

Candida albicans is an opportunistic pathogen which causes both superficial and systemic infections in mammals (29). It is generally recognized that adhesion of *C. albicans* directly to host surfaces or to plastic prostheses (e.g., indwelling catheters and dentures) is a crucial initial step in colonization and pathogenesis (18, 33). Various investigations have been carried out to determine the nature of the adhesins which mediate the initial attachment of *C. albicans* to host cells as well as to plastic surfaces (5, 11). Some studies have invoked the importance of hydrophobic interactions in mediating adhesion of *C. albicans* to a variety of surfaces (13, 20, 22). Other reports suggest that adhesion is mediated by stereospecific interactions (5). Potential adhesins include mannoproteins (6), mannans (28), lipids (9), and chitin (38).

One way of further elucidating the nature of the adhesin(s) involved in binding of *C. albicans* is by taking a genetic approach. However, classical techniques employed for bacteria to resolve similar problems (e.g., selection of mutants) are inappropriate for *C. albicans* because of its diploid state and the lack of sexuality. Recently, new genetic techniques have been developed to overcome this problem (for reviews, see references 23 and 35). One promising approach is based on the observed expression of *C. albicans* genes cloned in *Saccharomyces cerevisiae* that complement functionally known mutations (31) or confer new attributes to the recipient (8). In the present study, we followed such an approach in an attempt to identify *C. albicans* genes related to adhesion. Adherent transformants were selected from a population of nonadherent *S. cerevisiae* cells harboring a *C. albicans* genomic library. An adherent transformant which exhibited enhanced adhesion to polystyrene and epithelial cells as well as autoaggregation properties was isolated. The DNA fragment responsible for these properties was partially characterized.

MATERIALS AND METHODS

Strains and plasmids. The strain that served as the source for the genomic library was *C. albicans* B792 (B serotype), originally isolated and characterized by Kwon-Chung and Hill (24). The high-copy-number genomic library derived from this strain was previously described by Rosenbluh et al. (31). This library was made with the yeast-*Escherichia coli* shuttle vector YEp13 (4). The genomic library was transformed into the recipient yeast strain *S. cerevisiae* JWG2-11D (37), which is a multiple auxotroph for leucine and tryptophan. For subcloning, plasmids YEp351 and YEp352 were used (15). *E. coli* DH5α was used in transformations and for amplification of the cloned DNA.

Media and growth conditions. For *C. albicans*, complex medium YPD, containing 1% yeast extract, 2% peptone, and 2% glucose, was used. *S. cerevisiae* strains were grown on minimal medium SD, consisting of 0.67% yeast nitrogen base (without amino acids), 2% glucose, and appropriate amino acids, as described by Sherman et al. (36). *E. coli* strains were grown in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) supplemented with ampicillin (50 μg/ml) for plasmid maintenance. Yeast strains were grown at 28°C; *E. coli* strains were grown at 37°C.

DNA manipulation. Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs (Beverly, Mass.) and used according to the manufacturer's

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instructions. DNA fragments were analyzed on 1% horizontal agarose gels. Plasmid DNA was isolated from *E. coli* by the method of Birnboim and Doly (2). Plasmids were isolated from yeasts by the method described by Hoffman and Winston (16).

Transformation procedures. *S. cerevisiae* was transformed by the lithium acetate method of Ito et al. (17). *E. coli* was transformed by using commercially obtained competent *E. coli* DH5α cells (BRL Life Technologies, Gaithersburg, Md.) according to the manufacturer's instructions.

Southern blot analysis. Southern blots were prepared with genomic DNA isolated from yeast strains as described by Hoffman and Winston (16) and transferred to nitrocellulose filters (39). ^{32}P-labeled probes were prepared and used as described by Maniatis et al. (26).

Selection procedure. In preliminary experiments (not shown), washed suspensions of *C. albicans* B792 were found to adhere more avidly to polystyrene (tissue culture treated by electronic introduction of negative surface charge, according to the manufacturer) than *S. cerevisiae* JWG2-11D. For selection of adherent transformants, an agar overlay method was used (25). Cells of *S. cerevisiae* JWG2-11D harboring the *C. albicans* gene library were washed and suspended in TE buffer (10 mM Tris-HCl, 0.5 mM EDTA [pH 7.4]) to a concentration of ca. 3×10^7 CFU/ml. Samples (0.8 ml) were added to 3.5-cm-diameter tissue culture polystyrene plates (NUNC, Roskilde, Denmark). Plates were allowed to stand for 1 h at room temperature. After extensive washing in water, the plastic surface was overlaid with 0.8 ml of selective solid growth medium, consisting of SD supplemented with tryptophan and devoid of leucine, to select adhering transformants. After 72 h of incubation at 28°C, a number of colonies were detected and tested for adhesion and aggregation properties. One transformant, TY21, exhibited pronounced adhesion and aggregation and was selected for further characterization.

Adhesion to polystyrene. Adhesion to polystyrene was tested as described previously, with several modifications (7). Yeast cells were washed twice and suspended in TE buffer to an optical density of 9 optical density (OD) units (400 nm, 1-cm light path). Samples (150 μl) were added to flat-bottomed wells of either tissue culture-treated or untreated polystyrene (Cel-Cult; Sterilin, Feltham, England, and Sero-Wel, Sterilin, Hounslow, England, respectively) already containing 50 μl of TE (to prevent uneven initial adhesion). Plates were shaken vigorously on a plate shaker (Titertek, Flow Laboratories, Irvine, Calif.) for 1.5 h. Wells were washed gently by adding and removing 100 μl of TE buffer 20 consecutive times, taking care to prevent drying of the substratum. Adhering cells were then stained by adding 100 μl of 1% gentian violet to each well and shaken for 15 min. The plates were then rinsed 10 times with running water and allowed to dry. Adhesion was measured as the A_{610} with a microtiter plate reader (Bio-Tek Instruments, Burlington, Vt.).

Autoaggregation. Autoaggregation was assessed qualitatively by visual and microscopic observation of cell suspensions. For quantitative measurement, yeast cells were washed twice and suspended in TE buffer to an optical density of ca. 8 OD units (400 nm, 1-cm light path). Yeast suspensions (0.5 ml) were added to 0.5 ml of double-strength yeast nitrogen base (Difco; 1.34%) in TE buffer, and the optical density was measured. Following 1 h of horizontal agitation in 5-ml test tubes on a reciprocal shaker (at 350 rpm), suspensions were transferred to 1-ml disposable cuvettes and optical density was read both immediately and after 60 min. Autoaggregation was measured as the percent decrease in optical density at 400 nm (OD_{400}) after agitation and settling compared with that of the suspension prior to agitation.

Adhesion to human buccal epithelial cells. Human buccal epithelial cells were collected by gently rubbing the inside cheek area with sterile swabs and swirling the swabs in PBS (8 g of NaCl, 0.2 g of KCl, 1.15 g of Na_2HPO_4, 0.2 g of KH_2PO_4, and distilled water to 1 liter [pH 7.2]). The cells were washed twice and suspended in PBS to a final concentration of 2×10^5 CFU/ml. Yeast cells were grown as described, washed twice, and suspended in PBS to a concentration of 10^8 CFU/ml. Adhesion was carried out essentially as described by Sandin and Rogers (34). Briefly, 0.25-ml samples of yeast cells and buccal epithelial cells were combined in 10-ml test tubes, which were shaken horizontally on a rotary shaker (200 rpm) for 2 h at room temperature. Polycarbonate filters (12-μm pore size; Poretics Corp., Livermore, Calif.) were used for filtration of the mixtures. Filters were washed with 70 ml of PBS under vacuum and allowed to dry. Filters were fixed with absolute methanol and stained with 1% gentian violet. The number of yeasts adhering to each of 100 buccal cells was determined by light microscopy at a magnification of 400×.

RESULTS

Selection and characterization of adherent transformants. Selection of the adherent transformant among *S. cerevisiae* JWG2-11D cells harboring the *C. albicans* genomic library on YEp13 was carried out as described in Materials and Methods. After incubation for 72 h, individual colonies were characterized to determine their genotype to verify that the colonies were in fact putative transformants and tested for adhesion and autoaggregation properties. One transformant (TY21) exhibited much higher adhesion to treated and untreated polystyrene, as well as observed autoaggregation, than *S. cerevisiae* JWG2-11D cells bearing the vector alone. Growth of TY21 on complex medium (with no selection for the plasmid) resulted in loss of the adhesion and autoaggregation properties concomitant with loss of the plasmid, as reflected by reversion of the transformant to auxotrophy for leucine. This suggested that the adhesion and autoaggregation phenotypes were plasmid-linked traits. Furthermore, of 68 randomly chosen *S. cerevisiae* clones from the genomic library, none were observed to exhibit enhanced autoaggregation.

Plasmid DNA was isolated from TY21 and transformed into *E. coli* for DNA isolation and manipulation. The plasmid recovered was YEp13 with an insert of 4.5 kb, within the expected range present in the original *C. albicans* genomic library employed (31). The isolated plasmid was used for a second round of transformation in *S. cerevisiae* and found to confer the LEU phenotype as well as adhesion and autoaggregation on all the transformants tested.

The restriction analysis of pTY21 is summarized in Fig. 1. Following restriction with *Bam*HI, the insert was reduced to 3.3 kb (yielding pTY21-1). *S. cerevisiae* cells bearing this reduced insert were tested for adhesion and aggregation phenotypes by three methods: (i) adhesion to tissue culture-treated and untreated polystyrene (Table 1); (ii) autoaggregation in yeast nitrogen base (Table 2 and Fig. 2); and (iii) adhesion to buccal epithelial cells (Table 3). The results showed that cells bearing pTY21-1 exhibited significantly greater adhesion to all surfaces than the cells without the insert. For example, adhesion of *S. cerevisiae* cells bearing

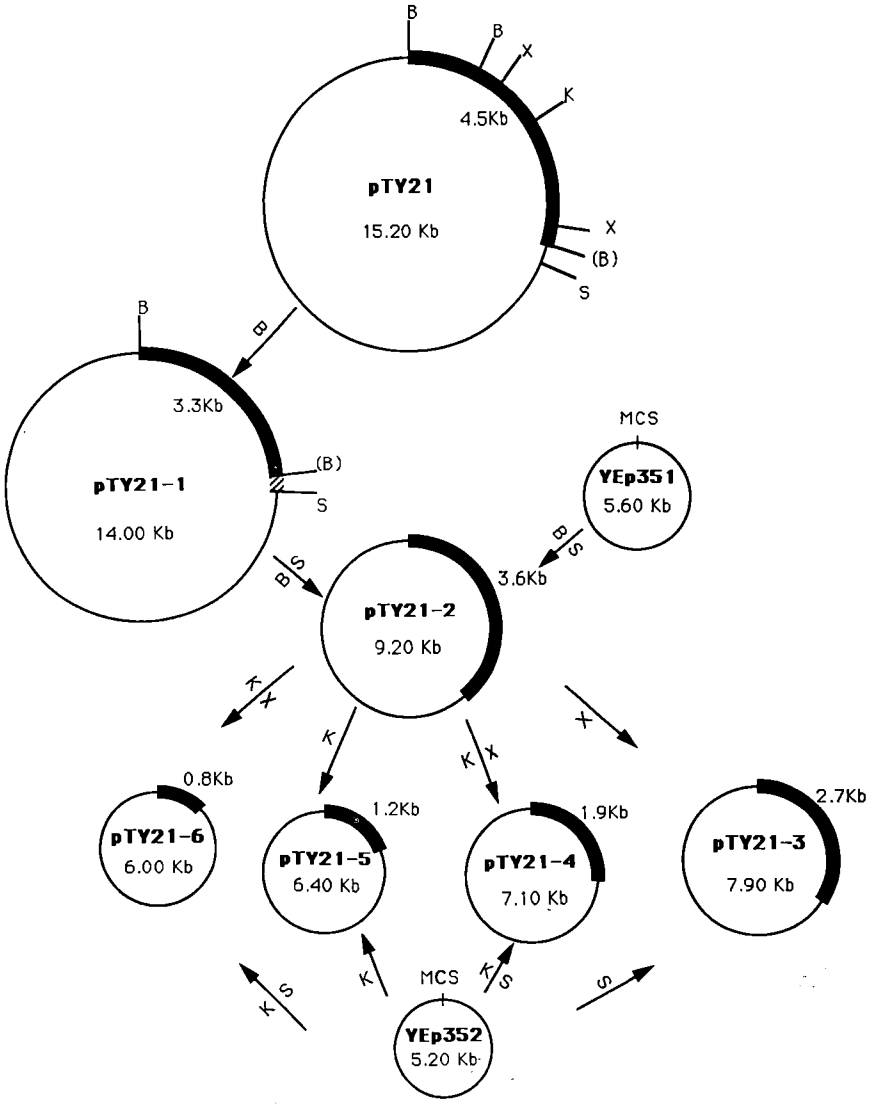

FIG. 1. Schematic diagram of steps in the construction of the genomic subclones used to identify the adhesion and autoaggregation region. Plasmid pTY21, containing a 4.5-kb fragment inserted into YEp13, was isolated from the genomic library as described in Materials and Methods. The thick black bars represent the portion of the original 4.5-kb fragment inserted into the various subclones. The arrows and adjacent abbreviations indicate the restriction endonucleases used to construct each subclone. Abbreviations: B, *Bam*HI; S, *Sal*I; X, *Xho*I; K, *Kpn*I, MCS, multiple cloning site.

pTY21-1 to treated and untreated polystyrene was 69 and 14 times greater, respectively, than that of *S. cerevisiae* cells with the vector (YEp13) alone (Table 1).

Results similar to those found for adhesion to polystyrene were also observed for autoaggregation (Table 2 and Fig. 2). Cells were agitated for 1 h to facilitate aggregation. They were then placed in cuvettes for measurement of optical density and read immediately (time zero) and after 1 h. The transformant bearing pTY21-1 was observed to be highly autoaggregative, reaching 46 and 77% reduction in turbidity immediately and 60 min after agitation, respectively, compared with 14% for *S. cerevisiae* with YEp13 alone.

Transformants were observed to undergo considerable aggregation during growth on SD medium containing yeast nitrogen base. It was of interest to attempt to determine which of the component factors might be involved in the aggregation process. Table 4 presents the results of an

TABLE 1. Adhesion of yeast cells to tissue culture-treated and untreated polystyrene[a]

Strain and plasmid	Adhesion to polystyrene (10^2 OD units)	
	Tissue culture treated	Untreated
C. albicans B792 (none)	1.5 ± 3.8	15.7 ± 6.2
S. cerevisiae JWG2-11D		
pTY21-1	172.5 ± 58[b]	110.0 ± 8.9[b]
YEp13	2.5 ± 0.9	8.0 ± 3.5
None	−0.6 ± 2.1	10.2 ± 2.2
pTY21-2	68.5 ± 6.4[c]	74.2 ± 9.5[c]
YEp351	4.5 ± 5.5	0.2 ± 1.2

[a] Adhesion to polystyrene wells was assayed as described in Materials and Methods. Results represent averages for four to six wells ± standard deviation.

[b] Significantly different ($P < 0.0005$) from value for *S. cerevisiae*(YEp13) (unpaired *t* test).

[c] Significantly different ($P < 0.0005$) from value for *S. cerevisiae*(YEp351) (unpaired *t* test).

TABLE 2. Autoaggregation of cells of *S. cerevisiae* JWG2-11D carrying various plasmids[a]

Plasmid	Aggregation (% decrease in turbidity)	
	Immediately after agitation	60 min after agitation
pTY21-1	46	77
YEp13	14	14
None	10	10
pTY21-2	47	73
YEp351	16	16

[a] Cell suspensions were aggregated by 60 min of agitation, as described in Materials and Methods. Results are expressed as the percent decrease in turbidity compared with the turbidity prior to agitation.

TABLE 3. Adhesion of yeast cells to human buccal epithelial cells

Strain and plasmid	% of epithelial cells bearing 1 or more attached yeast cells	Mean no. of yeast cells/epithelial cell[a]
S. cerevisiae JWG2-11D		
None	9	5
pTY21-1	49[b]	28[c]
YEp13	6	14
C. albicans B792 (none)	87[d]	42[d]

[a] Only epithelial cells bearing one or more yeast cells were included in the calculation.
[b] Significantly different ($P < 0.0001$) from adhesion values for the two *S. cerevisiae* controls (chi-square analysis).
[c] Significantly different from adhesion values for *S. cerevisiae* JWG2-11D ($P < 0.01$) and *S. cerevisiae* JWG2-11D(YEp13) ($P < 0.05$; unpaired t test).
[d] Significantly different ($P < 0.0001$) from adhesion values for transformant *S. cerevisiae* JWG2-11D(pTY21-1) (chi-square analysis).
[e] Significantly different ($P < 0.01$) from adhesion values for transformant *S. cerevisiae* JWG2-11D(pTY21-1) (unpaired t test).

experiment in which cells were rinsed either in double-distilled water or in TE buffer and then suspended in solutions containing various inorganic components of yeast nitrogen base. When the cells were washed in double-distilled water, ammonium sulfate was sufficient to duplicate the aggregation observed in yeast nitrogen base. However, when the cells were washed in TE buffer, ammonium sulfate alone had little effect unless other salts were present. Among the cations tested, calcium appeared to have the greatest effect on aggregation (Table 4).

The adhesion of transformant cells bearing pTY21-1 to buccal epithelial cells was significantly greater than that of *S. cerevisiae* cells carrying the vector YEp13 without the insert as well as that of untransformed cells of *S. cerevisiae* (Table 3). Adhesion of the donor *C. albicans* strain was significantly higher than that of the transformant. These differences were significant whether the percentage of epithelial cells bearing attached yeast cells or the mean number of yeast cells adhering per epithelial cell was compared.

Subcloning of the insert in other shuttle vectors. In order to facilitate further subcloning, the insert was transferred to YEp351, a vector that contains multiple cloning sites (15). A 3.6-kb *Bam*HI-*Sal*I fragment from pTY21-1 was subcloned in YEp351 (Fig. 1). Assuming that bacteria bearing the plasmids with the desired insert might yield colonies exhibiting altered morphological appearance, five abnormal colonies were examined. Among these transformants, two contained the 9.2-kb plasmid (pTY21-2) containing the previous 3.3-kb insert. DNA digestion with several restriction enzymes revealed two *Xho*I restriction sites and one *Kpn*I site (Fig. 1). Transformed *S. cerevisiae* cells bearing pTY21-2 exhibited the adhesion and aggregation phenotypes, whereas

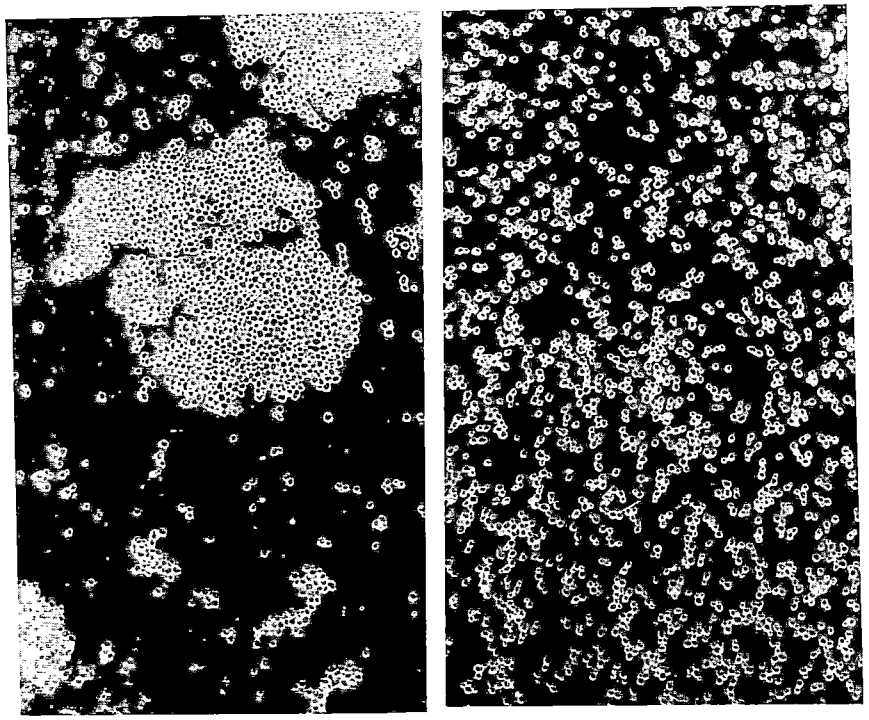

FIG. 2. Autoaggregation. Photomicrograph showing autoaggregation of *S. cerevisiae* transformants carrying pTY21-1 (left) or no plasmid (right). Magnification, ×160.

TABLE 4. Effect of washing with and addition of various compounds on aggregation of transformant TY21-1[a]

Washing and suspension compound	Addition(s) to suspensions[b]	Aggregation[c] (% decrease in turbidity)
Double-distilled water	None	0
	0.5% $(NH_4)_2SO_4$	69
	0.5% $(NH_4)_2SO_4$ + 0.01% $CaCl_2$ + 0.01% NaCl + 0.05% $MgSO_4$	68
	YNB	67
TE buffer	None	10
	0.5% $(NH_4)_2SO_4$	12
	0.01% $CaCl_2$	30
	0.01% NaCl	21
	0.05% $MgCl_2$	15
	0.5% $(NH_4)_2SO_4$ + 0.01% $CaCl_2$ + 0.01% NaCl + 0.05% $MgSO_4$	44
	YNB	62

[a] Cell suspensions were aggregated by agitation as described in Materials and Methods. Results are expressed as the percent decrease in turbidity compared with that of the suspension prior to agitation.
[b] Added to suspensions after washing (final concentrations). YNB, yeast nitrogen base.
[c] Measured immediately after the 60-min agitation.

cells bearing only the YEp351 vector did not (Tables 1 and 2).

In order to take advantage of the *Kpn*I cleavage site within the insert, a second plasmid, YEp352 (15), was used because this plasmid has a unique *Kpn*I site located in the multiple cloning site. YEp352 differs from YEp351 in that it possesses a *URA3* gene as a selection marker rather than the *LEU* gene. For selection of YEp352, strain JWG2-11D was converted by gene transplacement (32) to uracil auxotrophy. *Ura3* auxotrophs were identified as being resistant to 5-fluoro-orotic acid (3). The transplacement of *URA3* was confirmed by transformation with YEp352, which restored the prototrophic phenotype to JWG211-D.

A series of subclones were constructed by digestion of pTY21-2 and YEp352 with restriction endonucleases and ligation of the desired combinations, yielding the following subclones (Fig. 1). (i) pTY21-3 carried an *Xho*I-*Xho*I 2.7-kb fragment inserted into YEp352 which had been cleaved by *Sal*I; (ii) pTY21-4 carried a *Kpn*I-*Xho*I fragment of 1.9 kb, inserted into YEp352 that had been cleaved by *Kpn*I and *Sal*I; (iii) pTY21-5 carried a *Kpn*I-*Kpn*I 1.2-kb fragment (one cleavage site within the insert and a second in the MCS), inserted into YEp352 which had been cleaved by *Kpn*I; and (iv) pTY21-6 carried a *Kpn*I-*Xho*I fragment of 0.8 kb, inserted into YEp352 that had been cleaved by *Kpn*I and *Sal*I. Transformed *S. cerevisiae* JWG2-11D *ura3* cells bearing the various subclones were tested for adhesion and autoaggregation phenotypes and compared with JWG2-11D *ura3* cells bearing pTY21-2 (Tables 5 and 6). The data show that reduction of the insert size below 3.3 kb results in incremental losses in adhesion and aggregation. For example, adhesion of the 2.7-kb fragment in YEp352 (pTY21-3) to tissue culture-treated and untreated polystyrene was 99 and 47% of that of the 3.3-kb fragment, respectively. Autoaggregation of cells bearing the 2.7- and 1.9-kb fragments was 81 and 38%, respectively, of that exhibited by the 3.3-kb fragment. Cells

TABLE 5. Relative adhesion of *S. cerevisiae* JWG2-11D *ura3* carrying various subclones to tissue culture-treated and untreated polystyrene[a]

Plasmid	Adhesion to polystyrene (10^2 OD units)	
	Tissue culture treated	Untreated
pTY21-2	115.0 ± 19.7	112.0 ± 21.6
pTY21-3	114.0 ± 21.0[b]	52.8 ± 23.9[b]
pTY21-4	66.6 ± 28.3[c]	22.4 ± 5.4[b]
pTY21-5	15.2 ± 2.3	−5.0 ± 7.1
pTY21-6	10.8 ± 2.3	−8.7 ± 10.0
YEp352	11.2 ± 8.7	−6.8 ± 7.5
None	4.3 ± 7.3	−0.4 ± 3.4

[a] Adhesion to polystyrene wells was assayed as described in Materials and Methods. Results represent averages for four to six wells ± standard deviation.
[b] Significantly different ($P < 0.0005$) from value for *S. cerevisiae* JWG2-11D(YEp352) (unpaired *t* test).
[c] Significantly different ($P < 0.0025$) from value for *S. cerevisiae* JWG2-11D(YEp352) (unpaired *t* test).

bearing shorter fragments of 1.2 and 0.8 kb did not show the adhesion and autoaggregation phenotypes (Tables 5 and 6).

Hybridization of the 2.7-kb DNA fragment with *C. albicans* genomic DNA. To confirm that the DNA fragment responsible for the adhesion and autoaggregation phenotypes originated in the *C. albicans* genome, hybridization experiments were carried out. Restricted DNAs from *C. albicans* B792 and *S. cerevisiae* JWG211-D were probed with the 2.7-kb *Xho*I fragment. The results of the hybridization are shown in Fig. 3, confirming that the 2.7-kb labeled fragment originated from *C. albicans* and that *S. cerevisiae* has no homologous sequence. Furthermore, the results indicate that *C. albicans* has a single copy of this gene. In addition, the 2.7-kb fragment was used to test for homology to the CR2 receptor recently cloned and characterized by R. A. Calderone (4a). The results show that the clone conferring adhesion and autoaggregation reported in this study is not homologous to the CR2 receptor.

DISCUSSION

Although many investigations on *C. albicans* adhesion have been reported, few studies have addressed the genetic aspects involved. In the present study, we have shown that a unique DNA segment from *C. albicans* can transform nonadherent *S. cerevisiae* cells to adherent and autoaggre-

TABLE 6. Autoaggregation of *S. cerevisiae* JWG2-11D *ura3* carrying various subclones[a]

Plasmid	Aggregation (% decrease in turbidity)	
	Immediately after agitation	60 min after agitation
pTY21-2	58	80
pTY21-3	21	65
pTY21-4	6	31
pTY21-5	0	0
pTY21-6	0	4
YEp352	0	0
None	0	2

[a] Cell suspensions were aggregated by 60 min of agitation, as described in Materials and Methods. Results are expressed as the percent decrease in turbidity compared with the turbidity prior to agitation.

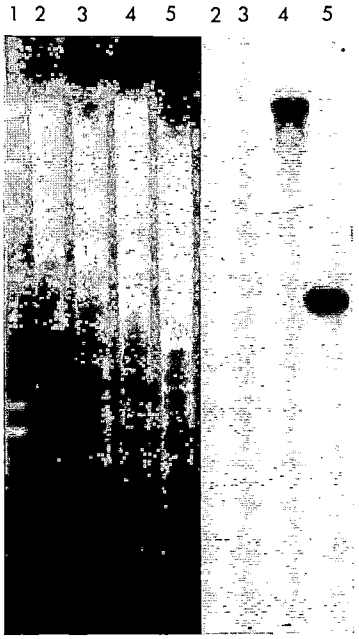

FIG. 3. Hybridization of subclone pTY21-3 to genomic DNA from *S. cerevisiae* JWG2-11D and *C. albicans* B792 digested with *Hin*dIII (lanes 2 and 4, respectively) and with *Eco*RI (lanes 3 and 5, respectively). Lane 1 is a *Hin*dIII digest of bacteriophage lambda. The gel-purified, ^{32}P-labeled, 2.7-kb fragment from the *C. albicans* insert was used as a probe. Left panel, ethidium bromide-stained agarose gel; right panel, autoradiograph of Southern hybridization.

gative phenotypes. Although adhesion to polystyrene was initially used for selection, it was subsequently found that the DNA segment concomitantly confers autoaggregation as well as adhesion to buccal epithelial cells. To the best of our knowledge, this is the first report of a candidal DNA segment which mediates adhesion to plastic as well as to host surfaces. Preliminary results (not shown) with rabbit antiserum prepared against the transformant and adsorbed with *S. cerevisiae* cells suggest the presence of a specific surface antigen(s) that is present on both transformant TY21-1 and *C. albicans* cells but absent from the *S. cerevisiae* control. These findings raise the possibility that the *C. albicans* DNA segment encodes a surface component which is not expressed in host *S. cerevisiae* cells and which may be related to adhesion in the donor strain. Alternatively, the sequence may encode a protein with a regulatory function related to adhesion. Work is under way to elucidate the DNA sequence of the segment studied here, to identify the gene product, and to study its expression in both the yeast and mycelial phases of *C. albicans*.

To date, several mechanisms of *C. albicans* adhesion have been proposed (19). Some investigators have stressed the role of hydrophobic interactions in mediating adhesion to host tissues and plastic devices in contact with the host (10, 22, 30). Potential hydrophobicity-associated surface components which have been implicated in candidal adhesion to plastic surfaces include proteins of 60, 68, 200, and >200 kDa (40) as well as several components of lower molecular masses (14). Other potential components include cysteine-rich, ca. 30-kDa "hydrophobins" which have been isolated from the outer surface of *Schizophyllum commune* and *Aspergillus nidulans* (41, 42) but have yet to be identified or characterized in *C. albicans*.

One unexpected result was the relatively greater adhesion of transformants to plastic and aggregation than of donor cells. This may be related to regulation of expression of the gene product. Whereas the *S. cerevisiae* cells may produce or overproduce the component in a constitutive manner, it would be expected that its production in donor cells would be carefully regulated, perhaps concomitant with morphological changes. Subcloning of the fragment to sizes below 3.3 kb led to incremental decreases in adhesion and aggregation levels, perhaps due to truncation of regulatory sequences that lead to a decrease in the level of expression.

The finding that transformant cells adhere to buccal epithelial cells raises the possibility that the gene product is involved in adhesion to host tissue. Further work is under way to test this hypothesis. The highest levels of adhesion to buccal epithelial cells were observed for the donor *C. albicans* cells, which contrasted with their relatively poor adhesion to plastic surfaces. Hazen et al. (12, 13) have recently shown that *C. albicans* strains exhibiting similar levels of hydrophobicity (based on binding of latex beads) differed quantitatively in their adhesion to host tissues, suggesting that hydrophobicity is one of several factors determining adhesion to host tissue.

Aggregative (flocculating) strains of *S. cerevisiae* are often used in fermentations, e.g., in the brewery industry (27). The observation in the present report that a foreign DNA fragment can induce aggregation is of interest in this regard. The molecular mechanisms responsible for yeast aggregation have yet to be completely elucidated, although hydrophobic interactions and divalent cations appear to be involved (1, 21). In the present study, aggregation was stimulated by the presence of ammonium sulfate. However, this stimulation appears to be dependent on the association of divalent cations with the cell surface. When the cells were washed in buffer containing EDTA, ammonium sulfate alone had no effect, whereas addition of cations (particularly calcium) yielded partial aggregation. Another question of interest is the relationship between aggregation and adhesion. Eli et al. (7) have shown that bacterial aggregates adhere better to polystyrene than nonaggregated suspensions. The transformant may prove an interesting model organism in helping to determine what changes in *S. cerevisiae* surface structure prompt nonaggregating cells to become highly aggregative ones.

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A *Candida albicans* Surface Antigen Mediating Adhesion and Autoaggregation in *Saccharomyces cerevisiae*

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In a previous study (M. Barki, Y. Koltin, M. Yanko, A. Tamarkin, and M. Rosenberg, J. Bacteriol. 175:5683–5689, 1993), a 3.3-kb DNA fragment from *Candida albicans* which confers adhesion and autoaggregation in *Saccharomyces cerevisiae* was isolated and partially characterized. In this report, evidence is presented that the adhesion-autoaggregation phenotype observed in *S. cerevisiae* cells transformed with the candidal DNA fragment is due to expression of a *C. albicans* surface antigen. Rabbit antiserum, prepared against transformant *S. cerevisiae* cells, was adsorbed with *S. cerevisiae* bearing the vector alone. Immunofluorescence micrography showed that the adsorbed antiserum bound to the surface of transformant *S. cerevisiae* cells as well as to *C. albicans* cells, but only marginally to the *S. cerevisiae* control. The adsorbed antiserum specifically inhibited autoaggregation of transformant cells. Further adsorption of the antiserum with transformant cells eliminated both inhibition and immunofluorescence. Autoaggregative activity and immunofluorescence of transformant cells were abolished following proteolytic treatment. Western blot (immunoblot) analysis of candidal extracts revealed that the absorbed antiserum recognized a major candidal antigen of ca. 30 kDa which was present on both yeast-phase and germ tube cells. The data suggest that the observed adhesion-autoaggregation phenotype is due to the presence of a specific candidal antigen on the outer surface of the transformant cells.

The cell wall of the opportunistic fungal pathogen *Candida albicans* is considered to play an important role in establishing the initial contact with host tissues and other surfaces (for reviews, see references 5, 10, and 23). During recent years, the potential roles of various surface components and adhesion mechanisms have been examined (15). Studies employing immunological approaches have demonstrated the expression of specific mannans and mannoproteins (6, 7, 21), as well as receptors for laminin, fibrinogen, fibronectin, and complement components, on the surface of *C. albicans* cells (3, 17, 22). In addition to stereospecific interactions between adhesins and receptors, physicochemical properties (primarily hydrophobic interactions) may also be involved in the observed adhesion patterns (9, 11, 13, 16).

Since *C. albicans* is a dimorphic fungus, exhibiting both yeast and mycelial phases, differences in expression of surface antigens concomitant with morphological changes in the cell wall may have a large effect on adhesion properties. Similarly, changes in growth media and conditions have also been shown to dramatically influence cell surface characteristics (25, 26).

One way of attempting to resolve the complexities alluded to above is by means of a genetic approach. In a previous study (1), an adherent transformant *Saccharomyces cerevisiae* clone, harboring a 3.3-kb DNA fragment from a *C. albicans* genomic library, was isolated. This transformant exhibited enhanced adhesion to polystyrene and buccal epithelial cells as well as pronounced autoaggregation. In the present study, a specific antibody preparation was employed to probe the outer surface of transformant and donor strains. The antibody preparation (i) inhibited autoaggregation, (ii) bound to the surface of transformant cells in a specific manner, and (iii) recognized a specific component of ca. 30 kDa in candidal cell surface extracts.

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MATERIALS AND METHODS

Yeast strains. The adherent transformant employed in the present study, TY21-1, is *S. cerevisiae* JWG2-11D (auxotrophic for leucine, histidine, and tryptophan) transformed with a 3.3-kb DNA segment from *C. albicans* B792 in shuttle vector YEp13 (1). The transformant is a subclone of TY21 (*S. cerevisiae* JWG2-11D bearing an insert of 4.5 kb) which was selected from a cell population transformed with a high-copy-number *C. albicans* genomic library on the basis of its adhesion to polystyrene (1). The donor *C. albicans* strain B792 and the recipient *S. cerevisiae* JWG2-11D served as controls.

Media and growth conditions. For *C. albicans*, a complex medium containing 1% yeast extract, 2% peptone, and 2% glucose (YPD medium) was used. *S. cerevisiae* strains were grown on a minimal medium (SD) consisting of 0.67% yeast nitrogen base without amino acids (YNB) and 2% glucose and supplemented with the appropriate amino acids as described by Sherman et al. (24). Yeast cells were grown at 28°C for 18 and 48 h for *C. albicans* and *S. cerevisiae*, respectively. Conversion of *C. albicans* blastospores to the mycelial phase was performed essentially as described by Hazen and Hazen (12). Briefly, following growth, *C. albicans* cells were washed twice with ice-cold saline. Cells (2×10^6) were inoculated into 2 ml of fetal calf serum and incubated at 37°C for 3 to 5 h with shaking. Test tubes were kept at 4°C for immunofluorescence studies and for the cell surface extraction procedure.

Antiserum. Antiserum against whole cells of TY21-1 was

raised in rabbits as follows: TY21-1 cells were washed twice in phosphate-buffered saline (PBS; 8 g of NaCl, 0.2 g of KCl, 1.15 g of Na_2HPO_4, 0.2 g of KH_2PO_4 [each per liter; pH 7.2]), and the cell density was adjusted to 10^8 cells per ml. Three injections of 1 ml each were applied subcutaneously at 2-week intervals. Blood was collected 1 week following the last injection. Preimmune serum was taken from the same rabbits prior to the first injection.

TY21-1 specific antibodies. To enrich for antibodies against unique TY21-1 surface antigens, antiserum was adsorbed onto *S. cerevisiae* JWG2-11D transformed with vector YEp13 alone (JWG2-11D + YEp13). Cultures of *S. cerevisiae* JWG2-11D+YEp13, grown in 1 liter of medium as described above, were washed twice in 0.15 M NaCl. The pellet was then suspended in 10 ml of 0.15 M NaCl and divided into five test tubes. Five milliliters of the antiserum was added to one of the tubes, and the suspension was incubated with gentle shaking for 3 h at 4°C. The cells were then centrifuged, and the supernatant was transferred to another tube containing fresh cell suspension. The procedure was repeated for all five tubes. As a control, the *S. cerevisiae* JWG2-11D+YEp13-adsorbed antiserum was further adsorbed with TY21-1 cells in the same manner.

Autoaggregation. Yeast cells were washed twice and suspended in TE buffer (10 mM Tris-HCl, 0.5 mM EDTA [pH 7.4]) to an optical density of ca. 9 (400 nm, 1-cm light path). Yeast suspensions (0.5 ml) were added to 0.5 ml of double-strength YNB (1.34%) in TE buffer or to TE buffer alone, and the optical density was measured. Following 1 h of horizontal agitation in 5-ml test tubes on a reciprocal shaker (at 350 rpm), suspensions were transferred to 1-ml disposable cuvettes, and optical density was read both immediately and after 60 min. Autoaggregation was measured as the percent decrease in optical density at 400 nm compared with that of the suspension prior to agitation.

Antiserum-mediated inhibition of autoaggregation. To determine the effect of antiserum on autoaggregation, 0.5 ml of cell suspension (optical density, ca. 9) was incubated for 30 min with 0.5 ml of (i) anti-TY21-1 antiserum prior to and following adsorption, (ii) antiserum raised against *Serratia marcescens* (2), and (iii) preimmune rabbit serum. Following incubation, cells were washed and suspended in 0.5 ml of TE buffer and tested for autoaggregation as described above.

Effect of proteolysis on autoaggregation. To check the effect of proteolytic enzymes on autoaggregation, 0.5 ml of cell suspension (optical density, ca. 9) was incubated for 5 min at 30°C (19) with vigorous shaking (180 rpm) with either 1 mg of trypsin, proteinase K, or heat-inactivated (20 min at 100°C) trypsin per ml. Following treatments, cells were washed and suspended in 0.5 ml of TE buffer and tested for autoaggregation as described above.

Immunofluorescence. Organisms were washed twice in PBS or in TE buffer, and the concentration was adjusted to 4×10^7 cells per ml. TY21-1-specific rabbit antiserum (100 μl, diluted 1:3) was added to 100 μl of cell suspension, and mixtures were incubated for 2 h with gentle shaking at room temperature. Cells were washed twice with the appropriate buffer, and fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (Sigma Chemical Co., St. Louis, Mo.; final dilution, 1:50) was added to the cells. Following 1 h of incubation as described above, samples were viewed with a Zeiss Axioplan D-7082 microscope and photographed with a Zeiss MC100 camera using Kodak TM400 film.

Preparation of cell surface extracts. Cell pellets were extracted essentially as described by McBride et al. (20). Cells were washed twice in 0.15 M NaCl, and their concentration was adjusted to 5×10^8 cells per ml. Two-milliliter aliquots of cell suspensions were centrifuged. The pellets were weighed, suspended 1:2 (wt/vol) in 2% sodium dodecyl sulfate (SDS) containing 5% β-mercaptoethanol, and boiled for 20 min. The mixtures were then centrifuged, and the supernatants were stored at −20°C for SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

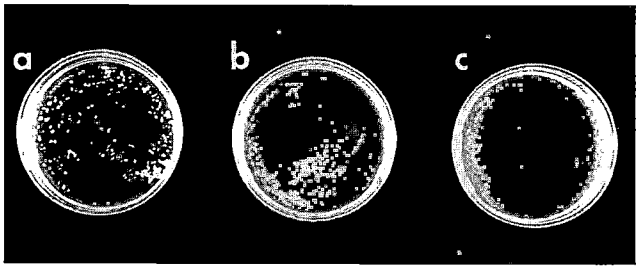

FIG. 1. Autoaggregation following growth. Following growth in appropriate media (see Materials and Methods), cell suspensions were transferred to petri dishes and photographed. (a) Transformant TY21-1; (b) *C. albicans* B792; (c) *S. cerevisiae* JWG2-11D+YEp13.

In some experiments, cell walls were digested by exposure to lyticase (a β1-3 glucanase; Sigma) as described previously (14). Briefly, yeast cells were washed three times in cold 0.15 M NaCl and suspended to 4×10^8 cells per ml of digest buffer (Tris hydrochloride [0.1 M, pH 8.1]) containing 1 mM phenylmethylsulfonyl fluoride. Cell wall digestion was initiated by mixing the cell suspension with an equal volume of lyticase solution (1,000 U/ml of digest buffer) and incubating it for 1 h at 35°C with occasional shaking. Following centrifugation (400 × *g*, 5 min), the supernatant was again centrifuged (10,000 × *g*, 10 min). Proteins from the second supernatant were precipitated with 80% (vol/vol) ethanol overnight at 0 to 4°C and pelleted by centrifugation (10,000 × *g*, 60 min). Following washing with cold (−20°C) ethanol, the pellet was dried, suspended in 10 mM Tris HCl buffer (pH 7.4), and stored at −20°C.

SDS-PAGE and Western blot (immunoblot) techniques. Antigenic extracts were dissolved 3:1 (vol/vol) in sample buffer containing 10% β-mercaptoethanol, and the mixtures were boiled for 3 to 5 min. Samples (45 μl) and standards (10 μl; molecular mass markers of 26 to 180 kDa [Sigma]) were loaded onto 10% polyacrylamide gels and electrophoresed as described by Laemmli (18). Gels were stained by a combined Coomassie brilliant blue and silver staining (8). Western blot analysis was performed as described by Burnette (4). Proteins were transferred to nitrocellulose paper and reacted with the TY21-1 specific antiserum.

RESULTS

Inhibition of TY21-1 autoaggregation by antiserum. Transformant TY21-1 undergoes dramatic, spontaneous autoaggregation during growth on SD medium (Fig. 1). Untransformed cells of *S. cerevisiae* JWG2-11D or cells transformed only with the vector (YEp13) are devoid of the autoaggregation phenotype. Autoaggregation of TY21-1 cells was shown to be due to the presence of ammonium sulfate and inorganic cations in the YNB component of the growth medium and is diminished following washing in TE buffer alone (1). On the basis of these findings, we developed a quantitative turbidimetric autoaggregation assay (1) in which washed cells are added to TE buffer containing YNB and agitated for 1 h as described in Materials and Methods.

TABLE 1. Effect of pretreatment with antisera on TY21-1 aggregation[a]

Medium	Antiserum	% Aggregation	
		Immediate	After 60 min
TE + YNB	None	72	77
	Anti-TY21-1 prior to adsorption	5	72
	Anti-TY21-1 following adsorption[b]	10	65
	Preimmune serum	68	68
	Anti-*S. marcescens*	67	73
TE	None	14	42
	Anti-TY21-1 prior to adsorption	17	75

[a] Following 30 min of incubation in the presence of antiserum, cells were washed and suspended in TE buffer containing YNB (TE + YNB) or TE buffer alone as indicated. Aggregation of cell suspensions was carried out by 60 min of agitation as described in Materials and Methods. Results are expressed as percent decrease in turbidity, measured immediately and then 1 h following agitation, as compared with the turbidity prior to agitation.

[b] Antiserum was subjected to repeated adsorption with suspensions of control *S. cerevisiae* cells as described in Materials and Methods. Adsorbed antiserum was diluted threefold as the result of the adsorption procedure.

TABLE 2. Effect of pretreatment with proteolytic enzymes on TY21-1 aggregation[a]

Medium	Enzyme treatment	% Aggregation
TE+YNB	None	80
	Trypsin	4
	Inactivated trypsin	79
	Proteinase K	4
TE	None	9

[a] Following 5 min of incubation in the presence of the indicated enzymes, cells were washed and suspended in TE buffer containing YNB (TE + YNB) or TE buffer alone as indicated. Aggregation of transformant cell suspensions was carried out by 60 min of agitation as described in Materials and Methods. Results are expressed as percent decrease in turbidity, measured immediately following agitation, as compared with the turbidity prior to agitation.

Table 1 summarizes a typical experiment showing the effect of antibody preparations on autoaggregation of transformant cells. Turbidity was monitored immediately and then 1 h following shaking. Cells suspended in TE plus YNB were highly aggregative, as shown by the 72% initial decrease in optical density. Almost complete initial inhibition of autoaggregation was observed in the presence of antibodies raised against TY21-1 both prior to and following adsorption with control *S. cerevisiae* JWG2-11D+YEp13 cells. Preimmune serum or antiserum raised against an unrelated bacterial species (*S. marcescens*) caused little or no inhibition of autoaggregation. As expected, cells suspended in TE buffer alone were less aggregative than cells incubated in the presence of YNB (Table 1). When suspensions were allowed to stand for 1 h following shaking, aggregation was observed in aliquots containing anti-TY21-1 antiserum (both prior to and following adsorption with *S. cerevisiae* JWG2-11D+YEp13). This can be attributed to antibody-mediated agglutination, since preadsorbed antiserum similarly stimulated clumping in the presence of TE alone (75% decrease in turbidity compared with 42% with no antibody present). As might be expected, when the anti-TY-21-1 *S. cerevisiae* JWG2-11D+YEp13-adsorbed antiserum was further adsorbed sequentially with transformant cells, the inhibitory effect was lost incrementally (data not shown).

Inhibition of TY21-1 autoaggregation by proteolytic enzymes. Table 2 shows the effect of proteolytic enzymes on TY21-1 autoaggregation. Trypsin and proteinase K destroyed the autoaggregative properties of the cells within 5 min of incubation. Inactivated trypsin had no effect on autoaggregation. As described below, pretreatment of transformant cells with trypsin similarly abolished their ability to bind TY21-1-specific antiserum.

Immunofluorescence. Immunofluorescence was carried out with anti-TY21-1 antiserum adsorbed on *S. cerevisiae* JWG2-11D+YEp13 cells. Antibody binding was visualized by fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G as described in Materials and Methods. Micrographs showing immunofluorescence of mycelial and yeast-phase donor *C. albicans* cells are shown in Fig. 2B and D, respectively, as compared with the same fields observed with Nomarski differential interference (Fig. 2A and C, respectively). Extensive binding of antibodies to both the mycelial and yeast phases of the donor *C. albicans* cells was observed. Micrographs showing immunofluorescence of transformant TY21-1 cells and control recipient *S. cerevisiae* JWG2-11D+YEp13 are presented in Fig. 3B and D, respectively, as compared with the same fields observed with Nomarski differential interference (Fig. 3A and C, respectively). TY21-1 cells were strongly fluorescent, whereas control *S. cerevisiae* JWG2-11D+YEp13 cells exhibited only background fluorescence. Whereas clumps of mycelial candidal cells and aggregates of transformants were highly immunofluorescent, individual hyphae and single transformant cells (e.g., following washings in TE buffer) were similarly fluorescent. Binding of antibody was specific, since fluorescein isothiocyanate-conjugated anti-rabbit IgG did not bind to any of the cells. Furthermore, when antiserum previously adsorbed to *S. cerevisiae* JWG2-11D+YEp13 cells was further sequentially adsorbed with transformant TY21-1 cells, gradual loss of TY21-1 immunofluorescence, concomitant with loss of inhibitory effect of the double-absorbed antiserum on autoaggregation, was observed (not shown). Finally, proteolytic treatment of transformant cells, with trypsin, abolished immunofluorescence as well as autoaggregation. It should be noted that background immunofluorescence of transformant and control cells was observed when both preimmune rabbit serum and rabbit antiserum raised against *S. marcescens* were

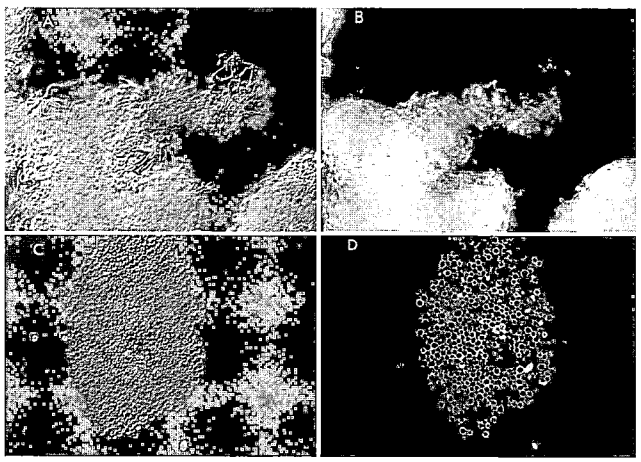

FIG. 2. Nomarski differential interference (A and C) and immunofluorescence (B and D) photomicrographs (magnification, ×230) of *C. albicans* cells reacted with antibody raised against TY21-1 and adsorbed with *S. cerevisiae* JWG2-11D+YEp13. Panels A and B show the same field of mycelium-phase cells. Panels C and D show the same field of yeast-phase cells.

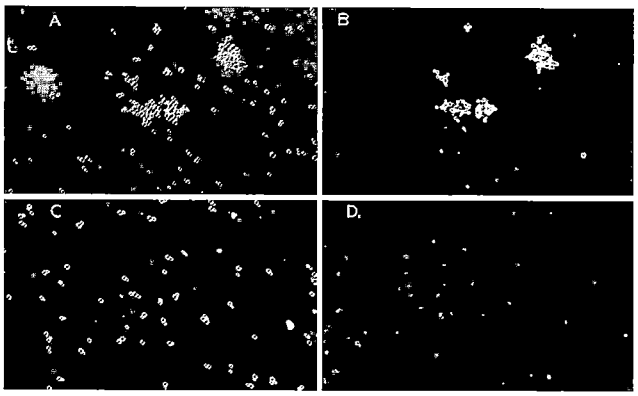

FIG. 3. Nomarski (A and C) and immunofluorescence (B and D) photomicrographs (magnification, ×125) of cells reacted with antibody raised against TY21-1 and adsorbed with *S. cerevisiae* JWG2-11D+YEp13. Panels A and B show the same field of transformant TY21-1 cells. Panels C and D show the same field of recipient *S. cerevisiae* JWG2-11D+YEp13 cells.

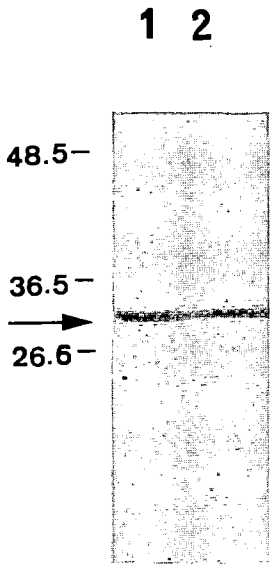

FIG. 4. Western blot of SDS–10% polyacrylamide gel loaded with cell surface extracts. Cells of donor *C. albicans* B792 grown as germ tubes (lane 1) and as yeast-phase cells (lane 2) were extracted as described in Materials and Methods and according to McBride et al. (19). Gel tracks were stained with the antibody raised against TY21-1 and adsorbed with *S. cerevisiae* JWG2-11D+YEp13. A strong band of ca. 30 kDa (arrow) is visible in both extracts. Molecular masses of standard proteins (in kilodaltons) are listed to the left of the gel.

used, presumably as a result of normal exposure of rabbits to yeast antigens. This immunofluorescence was diminished (but not totally eliminated) by adsorbing the preimmune serum with recipient *S. cerevisiae* JWG2-11D+YEp13 cells.

Western blot analysis of antigenic extracts. In an attempt to identify the antigen(s) associated with autoaggregation of TY21-1, cells were extracted as described in Materials and Methods, and the protein patterns were subsequently analyzed in SDS–PAGE and Western blots. Extracts of candidal cells (both yeast-phase cells and germ tubes), obtained following boiling in SDS–β-mercaptoethanol, yielded a major antigen of ca. 30 kDa which was visualized on Western blots by employing TY21-1-specific antibodies (Fig. 4). No such band, however, was observed in extracts of TY21-1 and control *S. cerevisiae* JWG2-11D+YEp13. Whereas several other bands were obtained in immunoblots of transformant and control cells extracted in the manner described above as well as by using the lyticase procedure, reproducible differences between transformant and control were not found (not shown).

DISCUSSION

In a previous report, we identified a DNA fragment from *C. albicans* that confers autoaggregation properties in *S. cerevisiae* cells (1). Transformant cells bearing this fragment similarly exhibited pronounced adhesion to tissue culture-treated and untreated polystyrene as well as to buccal epithelial cells. In the present study, we have provided evidence for a component on the outer surface of transformant *S. cerevisiae* cells which (i) is not observed in *S. cerevisiae* cells lacking the DNA fragment, (ii) may be related to a specific candidal surface antigen, and (iii) appears to mediate autoaggregation.

Antibodies specifically directed against the putative component were enriched for by raising antiserum against transformant TY21-1 and repeatedly adsorbing the antiserum with control *S. cerevisiae* cells possessing the vector YEp13 alone. The adsorbed antiserum specifically inhibited autoaggregation of transformant cells. This was due to binding of antibodies specific for the transformant outer surface, since further adsorption of the antiserum on transformant cells resulted in loss of the ability of the antiserum to inhibit autoaggregation.

In contrast to the initial anti-autoaggregation effect of the antiserum preparations, clumping of cells was observed when suspensions were allowed to stand for longer periods. This appears to be due to antibody-mediated agglutination since, in TE buffer (which does not promote aggregation), aggregation measured 1 h following agitation was much higher when antiserum was present.

To directly observe cell surface binding of the adsorbed antiserum preparation, fluorescent goat anti-rabbit immunoglobulin G was added to cells incubated in the presence of the TY21-1-specific antiserum. Microscopic observation indicated strong specific immunofluorescence of donor *C. albicans* and transformant cells, as compared with barely observable immunofluorescence of control *S. cerevisiae* cells. Preliminary data (not shown) suggest that the antigen may be associated with morphogenesis since immunofluorescence of the donor *C. albicans* cells is heavily concentrated along the developing germ tubes.

Antiserum, raised against transformant cells and adsorbed exhaustively by using control cells, recognized a specific antigen of ca. 30 kDa which was extracted from both yeast-phase and germ tube candidal cells. Interestingly, the same extraction procedure failed to yield an antigen of similar molecular weight from *Saccharomyces* cells, possibly as the result of strong anchorage of the component in the cell wall. In immunoblots of lyticase and SDS–β-mercaptoethanol extracts, several other common bands were observed in both *Saccharomyces* transformant and control cell extracts, possibly representing cryptic antigens. Work is under way to further determine whether the candidal antigen recognized by the specific antiserum is identical or similar to that appearing on transformant cells. This antigen may be related to the cysteine-rich ca. 30-kDa hydrophobins which have been isolated from the outer surface of *Schizophyllum commune* and *Aspergillus nidulans* (28, 29) but have yet to be identified or characterized in *C. albicans*. Other potential cell surface components which have

been suggested previously as participating in adhesion of other *Candida* strains to plastic surfaces include 60-, 68-, 200-, and >200-kDa proteins which were recovered from the substrata following cell desorption (27), as well as several other lower-molecular-weight components (14).

Taken together, the data suggest that the TY21-1-specific antibodies (i) recognize a trypsin- and proteinase K-sensitive outer surface component which is present on transformant but not on control *S. cerevisiae* cells, (ii) inhibit autoaggregation through specific binding to this component on the transformant cell surface, and (iii) recognize a specific antigen of ca. 30 kDa which is extractable from donor candidal cells. Work is under way to determine whether the transformed DNA fragment directly encodes this surface component and whether it acts as an adhesin in *C. albicans* cells.

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Relationship of Oral Malodor to Periodontitis: Evidence of Independence in Discrete Subpopulations

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ASSOCIATIONS BETWEEN ORAL MALODOR, measures of periodontal disease, and trypsin-like activity of periodontal pathogens on tongue and teeth were examined in 127 subjects. Volatile sulphur compound (VSC) measurements were made with a portable sulphide monitor; oral malodor was also estimated by organoleptic methods. Measurements repeated one week apart indicated that steady-state VSC levels ($r = 0.72$; $P = 0.0001$) and peak VSC levels ($r = 0.63$; $P = 0.0001$) were reproducible but these r values were not significantly different ($P > 0.1$). There was a significant correlation between tongue odor and peak VSC levels ($r = 0.40$; $P = 0.0001$) and between tongue odor and whole mouth organoleptic measures ($r = 0.55$; $P = 0.0001$). To study the effect of reducing microbial colonization on oral malodor, chlorhexidine gluconate (0.2%) rinsing was prescribed for 7 days. Reductions of VSC levels were significant for both peak (37%) and steady-state (41%) data ($P = 0.0001$). Anaerobic periodontal pathogens on the tongue estimated by the proportions of positive BANA tests were reduced 19% ($P = 0.001$) and this was concomitant with a 40% ($P = 0.0001$) decrease in organoleptic measurement of the tongue dorsum. Mean pH measurements of the tongue dorsum showed large reductions from 6.9 initially to 6.3 post-treatment ($P = 0.0001$). Subjects were divided into periodontitis/no periodontitis based on periodontal inflammation and probing depth (≥5 mm). Of the 37 subjects with periodontitis, 23 had oral malodor whereas 52 out of 90 periodontally healthy subjects exhibited malodor. Chi square analysis comparing halitosis in subjects with and without periodontitis showed no statistically significant association ($X^2 = 0.208$; P 0.65) between these two factors although the intensity of malodor as based on VSC concentration in periodontally healthy subjects was 19% less (mean = 111 ppb) than in subjects with periodontitis (mean = 136 ppb). The odds ratio was 1.2, indicating that oral malodor was not associated with periodontitis. These data indicate that a large proportion of individuals with oral malodor are periodontally healthy and that the mucosal surface of the tongue is a major site of oral malodor production. *J Periodontol 1994;65:37–46.*

Key Words: Chlorhexidine/therapeutic use; halitosis/etiology; tongue diseases/pathogenesis; sulfur compounds; periodontal diseases/pathogenesis.

Halitosis affects a large proportion of the population[1] and may be the cause of a significant social and psychological handicap[2] to those who suffer from it. Although little is known about the specific causes of oral malodor there are strong associations with periodontal diseases. For example, saliva from individuals with periodontitis putrefies more rapidly and the odor is more disagreeable in comparison to saliva from healthy individuals.[1,3,4] This is attributable in part to the degradation of blood and host cellular products that provide substrates for generation of volatile sulphur compounds (VSC) and that may in turn accelerate bacterial growth and proteolysis.[5] Conversely, uptake of volatile sulphurs by epithelial cells may play an important role in the pathogenesis of periodontal disease:[5] it has been proposed that volatile sulphurs may alter the permeability of affected cells and facilitate the access of toxic metabolites into the underlying connective tissue[5,6] thereby contributing to collagen degradation. There is also general agreement that the VSC content of exhaled mouth air[5,7,8] and the concentration of VSC precursors[9-11] increases with the severity of periodontal disease. Conversely VSC are reduced in subjects with good oral hygiene compared to those with gingivitis[12]

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and odor detected from dental floss is more intense after removal of mature plaque in comparison to nascent plaque.[13] Thus the presence of periodontal diseases and tooth-bound plaque appear to be strongly related to the detection of VSC in mouth air.

However, there also appears to be a distinct and separate population with detectable oral malodor that does not exhibit any periodontal disease.[11,14] Although the VSC content of mouth air in these individuals is not as high as those with periodontal diseases,[1,11,15] the breath of these individuals may be very offensive. Therefore, multiple etiologies of oral malodor independent of periodontal diseases and plaque are not only possible[16] but may represent a substantial proportion of the affected population. The objectives of this study were to examine quantitatively the association between oral malodor and periodontitis and to asses the relationship between measurements of VSC and bacteria on the tongue and dental sites.

MATERIALS AND METHODS

Subjects

Subjects were selected from two pools. First, all individuals who attended the Halitosis Assessment Clinic at the University of Toronto (n = 107) over the period from December 1, 1991 to June 21, 1992 were included. These subjects all reported halitosis as their chief complaint and had responded to a newspaper article on bad breath, to local and national television programs on this subject, or by referral from their dentist or physician. No exclusion criteria were used. Second, to ensure that subjects with a wide range of oral conditions were included, subjects without a chief complaint of halitosis were chosen at random from a large (n~2000) pool of staff, students, and patients at the Faculty of Dentistry (n = 26). This group was seen for one appointment only. The choice of sample size was based on convenience as there were inadequate previous data to provide accurate *a priori* calculations.

Subject Preparation

Subjects were asked to fill out a consent form and a medical history questionnaire. Conduct of this study was approved in advance by the Human Ethics Experimentation Committee at the University of Toronto. Subjects were asked to refrain from oral activities including eating, drinking, chewing, brushing, and mouth rinsing for 2 hours prior to each appointment. Subjects were also asked not to use commercial mouthrinse for one day prior to the appointment to reduce artifactual errors of volatile sulphur measurements associated with alcohol or zinc-containing mouthwashes.

Study Design

All subjects were measured at the first appointment to establish their baseline measurement. Only those subjects presenting to the Halitosis Assessment Clinic were measured at the second and third appointments which were held at intervals of approximately 1 week and at approximately the same time of day. For these subjects measurements from the second appointment were compared with those from the first appointment and assessed for reproducibility of the baseline data. Subjects were assessed by one of two evaluators who had been calibrated previously for organoleptic and periodontal measurements over a period of several clinical sessions prior to the study. To evaluate any differences in assessment each evaluator examined the same patients independently over several clinical sessions and the results were compared and the examiners calibrated.

Following the second appointment subjects were instructed to rinse with a 0.2% chlorhexidine gluconate (CHX) mouthrinse 2 times per day for a period of 1 week to assess any decrease in odor intensity due to microbial activity. Rinsing was done in the morning after breakfast and toothbrushing and in the evening just before bedtime. Each treatment consisted of brushing the tongue with a toothbrush soaked with CHX followed by a 60-second rinse and was performed from the evening of the second appointment to the evening of the third appointment, 1 week later. Rinsing with water following the CHX rinse was not encouraged. If subjects found the rinse too bitter, a period of 30 minutes waiting time was required prior to rinsing with water. Subjects were advised of possible side-effects due to CHX and asked to inform the principal investigator of these sequelae. All measurements were repeated on the third appointment and compared with the baseline data for any post-rinse differences.

Volatile Sulphurs

VSC measurements were made with a portable industrial sulphide monitor‡ zeroed on ambient air prior to each measurement. Two monitors, both with new sensors, were used and were calibrated for inter-monitor agreement (difference <5 parts per billion; ppb). This method of VSC measurement has been reported previously.[17,18] Briefly, each subject sat quietly without talking for 3 minutes prior to measurement of VSC levels. A disposable plastic straw was attached to the air inlet of the monitor. The subject was then instructed to bring his slightly open mouth over the straw so that it extended into the oral cavity approximately 4 cm. Subjects were then asked to breathe through the nose during the measurement. The sulphide monitor contained a pump which sucks air from the plastic straw placed inside the subject's mouth at approximately 1500 mL/min. As the sample of mouth air passed through an electrolytic sensor, the concentration of volatile sulphurs was esimated. Peak (maximum; VSCPK) and steady-state levels (VSCSS) were determined in ppb sulphur equivalents by direct reading from the analog scale of the monitor.

‡Model 1170, Interscan Corp., Chatsworth, CA.

Organoleptic Assessment

Subjects remained quiet and kept their lips closed for a period of 2 minutes. They were then asked to exhale through the mouth briefly with moderate force at a distance of approximately 10 cm from the nose of the evaluator. Following this, subjects exhaled through the nose for a nasal odor evaluation. Organoleptic and nasal malodor scores were estimated on a scale of 0 to 5 as follows: 0 = no odor; 1 = barely noticeable odor; 2 = slight but clearly noticeable odor; 3 = moderate odor; 4 = strong odor; and 5 = extremely foul odor. The reproducibility and validity of this method of oral malodor measurement have been previously reported.[18]

Tongue malodor was assessed by scraping the posterior tongue dorsum with gauze and immediately smelling its odor. The malodor was estimated using the same organoleptic scale. The amount of coating on the tongue dorsal surface was estimated by visual examination as heavy, medium, light, or none.

Malodor in dental sites was assessed by passing waxed dental floss interproximally between all teeth, smelling the floss immediately and recording the odor based on the organoleptic scale. Different segments of floss were used for each passage. The maximum organoleptic floss score and the mean value of sites with odor were used to assess the contribution of interproximal sites to oral malodor.

Periodontal Measurements

The probing depth of periodontal pockets was measured with a Michigan O probe to the nearest mm marking and recorded as > 5 mm or > 7 mm. Measurements of plaque index (PI)[19] and gingival index (GI)[20] were recorded, using the 6 Ramfjord sample teeth[21] at each appointment following malodor measurements.

A commercially available diagnostic test kit[§] for bacterial trypsin-like activity, based on the hydrolysis of benzoyl DL arginine-naphthylamide (BANA), was used to assess the presence of one or more of *T. denticola, P. gingivalis,* and *B. forsythus.* These species and some *Capnocytophaga* species possess an enzyme capable of hydrolysing the synthetic trypsin substrate, BANA, an activity not identified in other microbial species found in plaque.[22] The species identified by the BANA test have been tentatively implicated as putative periodontal pathogens and can also degrade proteins to volatile sulphur compounds.

In this study subgingival plaque samples from the mesial of first molars were obtained with an explorer and tested. The posterior surface of the tongue dorsum was scraped with a wooden tongue depressor and the scrapings were collected. Thick debris was discarded, the tongue was scraped again and the collected material was placed on strips that were incubated for 15 minutes.[22] A result which produced a definite blue to a pale blue color was recorded as positive. No reaction was recorded as a negative.

Salivary Measurement

Oral mucosal wetness was measured using paper strips and an instrument developed specifically for measurement of salivary flow rates.[‖] The accuracy of the unit in measuring small volumes of crevicular fluid has been determined previously.[23,24] The model we used has been adapted to measure larger volumes such as oral mucosal wetness and the flow of saliva. Measurements were made by placing small paper strips over the given area of the oral mucosa. For the purpose of this study paper strips were placed on the palate to measure flow from the palatal glands as well as the right and left posterior vestibular regions to measure parotid flow. These areas were dried with gauze prior to placement. Following a 5-second application, the strips were placed immediately into the sensor of the measuring unit. The displayed reading was converted to microliters using a standard calibration curve supplied by the manufacturer relating the readings to the volume of fluid (in microliters) absorbed by the paper strips.[#]

Litmus indicator paper was used to estimate the pH levels of the tongue dorsum, sublingual sites, and in the right and left posterior vestibules. One inch strips of pH indicator paper were placed on each of the surfaces and held until wet. The color was compared against standards and the pH was recorded.

Dental Measurements

The number of teeth, broken fillings, and caries were recorded. Fixed prostheses were examined for leaks and plaque and food impaction. Removable prostheses were taken out and measured organoleptically for fit and cleanliness. Lateral borders of the tongue, palate, and buccal mucosa were assessed for pathology and lesions. Desquamation, keratinization, and cheek biting were recorded. Pharyngeal tissues were checked for tonsillar enlargement and redness. Lip seal, mouthbreathing, and enlargement of lymph nodes were noted.

Statistical Analysis

The reproducibility of the VSC data were assessed by plots of differences against the means, by a paired *t*-test and by Pearson correlation. An analysis of covariance was used to determine systemic reduction in VSC values over time. Intra-examiner agreement was assessed by unweighted kappa statistic. The sensitivity of periodontal and malodor tests in detecting a reduction in values after CHX was assessed by paired *t*-tests. The Pearson correlation was used to assess

[§]Perioscan, Oral B, Redwood City, CA.

[‖]Periotron 6000, Model II, Interstate Drug, Amityville, NY.

[¶]Sialopaper, Pro Flow, Inc., Amityville, NY.

[#]pHydrion Vivid 6-8, Micro Essential Laboratory, Brooklyn, NY.

the strength of association between various malodor measurements and periodontal parameters. Backward stepwise regression analysis with the GLM (general linear model) procedure** was used to determine the malodor and periodontal parameters most strongly related to VSC. Positive BANA tests were counted separately for subjects with and without periodontitis and calculated as a percentage of the total number of tongue or tooth sites with a positive test.

RESULTS

One hundred and twenty seven subjects completed this study of whom 83 were female (43.2 ± 11.5 years) and 44 were male (43.1 ± 16.5 years). The duration of the study was 7 months. Of the 107 subjects from the Halitosis Clinic, 101 completed all three visits. Reasons for not attending all three sessions included unsuitability due to dental caries, long distances to travel, and failure to return for the last appointment. Data from these subjects were not included in the analyses.

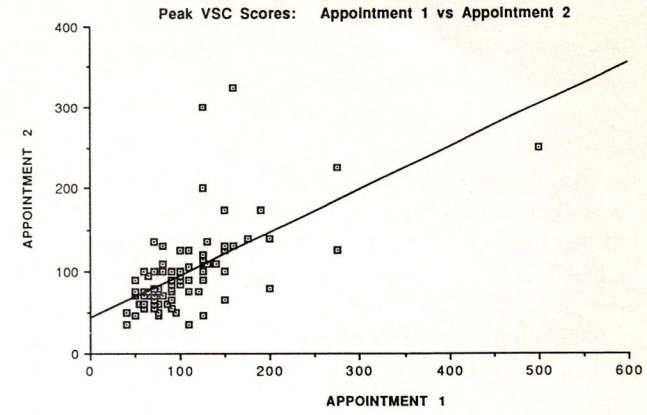
Of the 127 subjects who completed the study, only 2 were edentulous with complete upper and lower dentures. The dentate subjects had an average of 26.5 teeth and 13 of these subjects wore partial dentures. When the dentures were examined, only 1 full denture and 3 partial dentures had organoleptically measurable odor. All but 5 subjects reported regular dental examinations every 6 months or less. One subject had 2 carious lesions, 3 subjects had one caries each, and 1 subject had a broken filling. When the soft tissues were examined, 8 subjects exhibited minor pharyngeal inflammation, 2 had enlarged tonsils, and 4 subjects had some palatal inflammation. Seven subjects had deep fissures on the tongue dorsum and 2 had a geographic tongue. Seven subjects exhibited cheek biting and moderate desquamation on cheeks or edentulous areas. When checked for habits that may impact on their oral malodor, only 2 subjects were mouthbreathers and 9 were smokers.

Of the 101 subjects that were given CHX on the second appointment, 90 responded to a questionnaire on adverse reactions to the rinse. The most common reaction experienced by the subjects was the change in the taste of food (58.9%). A substantial number of subjects (25.6%) experienced discomfort in the form of a burning sensation on the tip of the tongue. Subjects also reported tongue dorsum staining (16.7%) and tooth staining (12.2%). Four subjects (4.4%) reported sloughing of tissues or gingival pain as a result of possible allergic reaction while only 11 subjects (12.2%) reported having no adverse reactions to rinsing with CHX.

Reproducibility

In contrast to our earlier study[18] we did not find a significant decrease in the sensitivity of measurements obtained from the monitor during the course of the study (6 months), indicating that the variations of VSCPK and VSCSS were due to biological responses and not due to instrument drift. Reproducibility measurements repeated one week apart (Fig. 1) indicated that steady-state VSC levels were more reproducible (r = 0.72; P = 0.0001) than peak VSC levels (r = 0.63; P = 0.0001) but this difference was not statistically significant. Organoleptic measurements (r = 0.72; P = 0.0001) between appointments 1 and 2 were as reproducible as steady-state VSC. A paired t-test showed no significant difference ($P > 0.1$) between appointment 1 and appointment 2 for peak VSC levels and for organoleptic scores. A significant difference ($P < 0.05$) between the two appointments for steady state VSC measurements was reduced to no difference ($P > 0.1$) when two extreme measures (difference of 70 and 75) were removed.

Intra-examiner agreement of whole mouth organoleptic measurements assessed with the unweighted kappa statistic indicated moderate consistency for whole mouth organoleptic measurement (examiner 1 = 0.48; examiner 2 = 0.54). Agreement of tongue dorsum scores was similarly



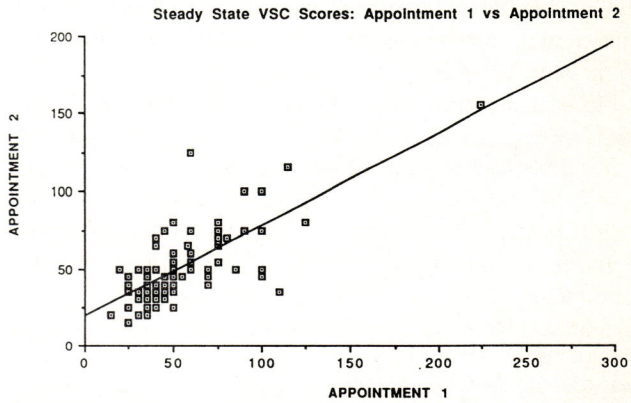


Figure 1. Scatter plots of peak and steady state VSC scores from first and second appointments showing the relationship between replicate measurements of VSC level. For steady state VSC, r = 0.72; for peak VSC, r = 0.63.

**SAS 6.03, SAS Institute, Cary, NC.

Table 1. Pearson Correlation Analysis of Periodontal and Malodor Measurements

VSCPK	VSCSS	Oral Odor	Nasal Odor	Tongue	GI	PI	Average Floss	Maximum Floss	Pockets* >5mm	Pockets* >7mm
VSCPK 1.000	0.815†	0.525†	0.287†	0.398†	0.036	0.039	0.109	0.198	0.143	0.107
VSCSS	1.000	0.444†	0.373†	0.367†	0.026	0.076	0.106	0.138	0.112	0-.070
Oral		1.000	0.148	0.549†	0.148	0.118	0.227†	0.202	0.108	−0.027
Nasal			1.000	0.210	0.028	0.152	0.021	0.101	−0.105	−0.087
Tongue				1.000	0.233†	0.037	0.125	0.168	0.054	0.155
Gingival index					1.000	0.259†	0.259†	0.292†	0.192†	0.113
Plaque index						1.000	0.234†	0.177	0.050	0.030
Average floss							1.000	0.590†	0.191	0.060
Maximum floss								1.000	0.287†	0.043
Pockets > 5 mm									1.000	0.441†
Pockets > 7 mm										0.1000

Pearson correlation coefficients/prob>|R| under Ho:Rho=0. Data from first appointment (all subjects) were analyzed. VSCPK=peak VSC scores; VSCSS=steady-state VSC scores; oral odor=whole mouth organoleptic scores; tongue odor=tongue organoleptic scores; GI=gingival index; PI=plaque index. Average floss=overall organoleptic scores for floss odor; maximum floss=maximum organoleptic scores for floss odors.
*Mean number of pockets/subject.
†Significant at $P > 0.01$.

analyzed and yielded Kappa values of 0.22 and 0.21 respectively.

VSC-Organoleptic Relationship

The relationship between whole mouth odor (measured organoleptically) and volatile sulphurs (measured by monitor) was assessed using data from the first appointment for all subjects. Peak VSC levels were more strongly associated with whole mouth organoleptic scores ($r = 0.53$; $P = 0.0001$) than with steady state VSC levels ($r = 0.44$; $P = 0.0001$; Table 1) but this difference was not statistically significant ($P = 0.18$). Nasal odors were weakly associated with peak VSC levels ($r = 0.37$; $P = 0.003$), and steady state VSC levels ($r = 0.37$; $P = 0.0001$) but not with whole mouth organoleptic scores ($r = 0.15$; $P = 0.14$).

Contribution of Oral Sites of Malodor Formation

Pearson correlation analysis (Table 1) of data from the first appointment was used to assess, specifically, the contribution of two separate intraoral loci to malodor formation. Tongue malodor assessed organoleptically was significantly correlated with peak VSC scores ($r = 0.40$; $P = 0.0001$), steady state scores ($r = 0.37$; $P = 0.0001$), and whole mouth organoleptic scores ($r = 0.55$; $P = 0.0001$) and increases in tongue malodor were directly proportional to the amount of coating present on the dorsal surface of the tongue ($r = 0.45$; $P = 0.0001$). Floss odors were assessed as an average score of at least 6 sites and secondly as the maximum, organoleptically-rated interproximal odor. The contribution of both these assessments to measures of oral malodor was small and of marginal significance. A backward step wise regression analysis of log transformed steady state VSC values from all subjects on the first appointment (Table 2) showed that the strongest associations of VSC were (in descending order), organoleptic scores ($P = 0.0001$), tongue odor ($P = 0.0001$), and maximum floss odor ($P = 0.02$).

Table 2. Backward Step Wise Regression Analysis of Log-Transformed Steady State VSC

Variable	Slope ± S.E.	F	Prob > F
Oral odor	0.173 ± 0.036	22.00	0.0001
Tongue odor	0.108 ± 0.04	6.78	0.0001
Maximum floss	−0.050 ± 0.022	5.07	0.0261

Overall regression equation - R-Square = 0.362; Intercept = 3.009 ± 0.126 F = 570.00 ($P = 0.0001$). Backward step-wise regression analysis with log-transformed steady state VSC values shown in descending order of the relative strength of associations of VSC.

Contribution of Bacteria to Oral Malodor

To examine the association of trypsin-like activity detected by the BANA test with oral malodor, BANA scores were compared to odors from teeth and tongue. The four tooth sites that were sampled showed a moderately strong relationship ($X^2 = 16.84$; $P = 0.001$) with floss odors. When related with tongue odor, the association of tongue dorsum BANA scores was comparatively weak ($X^2 = 6.52$; $P = 0.01$). There was no relationship between the presence of bacteria on the tongue dorsum and the presence of bacteria on teeth ($r = 0.08$; $P = 0.4$) using Pearson correlation. A calculation of positive test sites for all subjects showed that when periodontitis was present, 87.5% of tooth sites and 92.5% of tongue sites were BANA positive, while in healthy individuals, 74.4% of tooth sites and 94.3% of tongue sites were BANA positive.

Although oral dryness has been positively associated with oral malodor,[1] Pearson correlation assessment demonstrated no relationship between measures of oral wetness and oral measurement. This result was consistent with the results from a previous study.[18] There was also no relationship ($r = 0.02$; $P = 0.7$) between the pH of the tongue dorsum and the BANA test scores. Further, when the tongue pH values of healthy individuals were compared to those with periodontitis there was no detectable difference.

To study the effect of reducing the overall oral microbial

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Table 3. Comparison of Malodor and Periodontal Variables for All 3 Appointments (n = 101)

Variable	Appointment 1	2	3	% Change*
VSC PK	103.8±5.8	95.5±4.9	65.8±1.5	36.6
VSC SS	54.3±2.8	50.5± 2.3	32.0±1.9	41.1
Oral odor	2.9±0.1	2.8±0.01	1.0±0.1	65.5
Nasal odor	0.7±0.1	0.8±0.1	0.3±0.1	57.1
Tongue odor	2.5±0.1	2.6±0.05	2.5±0.1	40.2
Tongue coat	2.7±0.1	2.6±0.1	2.0±0.1	25.9
Gingival index	1.2±0.1	1.2±0.1	1.0±0.6	16.7
Plaque index	1.7±0.1	1.6±0.1	1.0±0.1	41.2
Floss odor*	3.3±0.3	3.0±0.2	2.1±0.2	36.3
Pockets ≥5 mm	0.8±0.1	0.8±0.1	0.6±0.2	25.0
Pockets ≥7 mm	0.2±0.1	0.2±0.1	0.2±0.2	0.0
pH Dorsum	6.9±0.1	7.0±0.1	6.3±0.1	400

Measurements are mean ± SE. Comparison of malodor and periodontal measurements for all 3 appointments and the percent change between the first and third appointment.
* Significant at $P = 0.0001$ except for gingival index, where $P = 0.05$ and for periodontal pockets where there was no change.

load on levels of oral malodor, CHX (0.2%) rinsing was prescribed for 7 days. When post-rinse measurements were compared to those obtained at the first appointment, large and significant ($P = 0.0001$) reductions of peak VSC levels (36.6%), steady state VSC levels (41.1%), and organoleptic scores (65.5%) were found (Table 3). Plaque indices were reduced by 41.2% ($P = 0.0001$) and there was a 25% reduction in the number of 5 mm and 6 mm pockets ($P = 0.0001$). The number of deep periodontal pockets (≥7 mm) did not change, however, the mean pH measurements of the tongue dorsum were significantly reduced ($P = 0.0001$; pH 6.9 to 6.3 before and after CHX treatment). Gingival indices were reduced by 16.7% ($P = 0.05$). Although this reduction was statistically significant, it was considerably less than the value ($P = 0.0001$) for all other reductions. There was a dramatic reduction in the proportion of BANA positive reactions in samples taken from tooth sites of healthy individuals (to 35.5%) and a smaller reduction in positive reactions of tongue sites (to 75% of total sites). In individuals with periodontitis, post-CHX BANA positive tooth sites were decreased to 54.7% and positive tongue sites to 71%.

Relationship of Measures of Periodontal Disease to Oral Malodor

For this study, plaque indices, gingival indices, and the mean number of pockets ≥5 mm and ≥7 mm per subject were used as measures of periodontitis. The association of these measures with VSC levels and organoleptic scores were assessed at the first appointment by Pearson correlation analysis. Plaque indices and gingival indices were not associated with VSC levels and only weakly with organoleptic scores. The presence of pockets was also not associated with VSC levels or organoleptic scores.

To examine the relationship between oral malodor and periodontal diseases from another perspective, all subjects were classified into 4 groups based on the presence of periodontitis and halitosis (Table 4). The presence of periodontitis was defined as the clinical detection of 1 or more pockets of ≥5 mm. Although malodor has been measured by several ordinal scales,[15,18] no standardized recognition value has been established for any measurement method. Based on data from a previous study[18] and by inspection of preliminary data, the presence of halitosis was defined in this study as an organoleptic measurement of ≥3. This threshold value was computed by subtracting the 95% confidence limit of the mean from the mean organoleptic score for the whole population.[18] The rationale for the application of this threshold was to include all subjects with easily detected oral malodor and to eliminate any subject with only mildly objectional levels. When the groups were compared with this dichotomous approach, only 23 subjects exhibited both periodontitis and organoleptic scores above the threshold (Table 5). Fifty-two subjects exhibited organoleptic scores above the threshold but no periodontitis; these individuals comprised the largest subpopulation. The second largest group had no periodontitis and organoleptic scores which were well below the threshold limit. A small group of 14 subjects with periodontitis had very low organoleptic scores. Chi square analysis comparing halitosis in subjects with and without periodontitis showed no statistically significant association ($X^2 = 0.208$; $P = 0.65$) between malodor and periodontitis. The odds ratio was 1.2 indicating that oral malodor was not associated with periodontitis.

Fisher's exact test was conducted to determine if associations between oral malodor and periodontitis were present in subjects (n = 26) recruited through sources other

Table 4. Dichotomous Analysis of Subjects Based on Halitosis and Periodontitis

	Periodontitis	No Periodontitis	Total
Halitosis	N = 23 (18.1%)	N = 52 (40.9%)	N = 75 (59.0%)
No Halitosis	N = 14 (11.1%)	N = 38 (29.9%)	N = 52 (41.0%)
Total	N = 37 (29.1%)	N = 90 (70.9%)	N = 127

Presentation of numbers of subjects in groups with and without halitosis and periodontitis based on criteria described in Materials and Methods.

Table 5. Malodor and Periodontal Parameters for Groups With and Without Periodontitis

Variable	Periodontitis/ Halitosis	No Periodontitis/ Halitosis	Periodontitis/ No Halitosis	No Periodontitis/ No Halitosis
Age (years)	50.5± 2.9	40.8±1.9	49.6±3.7	39.6±1.6
VSC Peak (ppb)	135.7±18.8	110.4±6.3	73.9±8.1	69.1±3.3
VSCSS (ppb)	67.6± 8.3	56.2±3.2	36.4±3.5	39.1±2.6
Oral score	3.5± 0.1	3.4±0.1	1.3±0.2	1.7±0.1
Tongue odor	2.7± 0.2	2.7±0.1	1.7±0.2	1.7±0.1
Tongue pH	6.9± 0.8	6.9±0.1	6.9±0.2	6.9±0.1
Pockets ≥5 mm	3.1± 0.6	0.0	2.0±0.4	0.0
Pockets ≥7 mm	0.4± 0.2	0.0	0.7±0.3	0.0
Gingival index	1.5± 0.1	1.2±0.08	1.4±0.1	1.0±0.1
Plaque index	1.9± 0.1	1.8±0.07	1.6±0.1	1.5±0.1

Measurements of malodor and periodontitis (mean and standard error) for each of the 4 groups identified in Table 4.

Table 6. Dichotomous Analysis of the Subset Based on Halitosis and Periodontitis

	Periodontitis	No Periodontitis	Total
Halitosis	N = 3 (11.5%)	N = 3 (11.5%)	N = 6 (23.0%)
No halitosis	N = 7 (26.9%)	N = 13 (50.0%)	N = 20 (76.9%)
Total	N = 10 (38.5%)	N = 16 (61.5%)	N = 26

Presentation of numbers of subjects other than those self reporting oral malodor in groups with and without halitosis and periodontitis based on criteria described in Materials and Methods.

than the oral malodor clinic (Table 6). Three subjects had both oral malodor and periodontitis; 3 subjects had oral malodor without periodontitis; 7 subjects had periodontitis without malodor; and 13 subjects had no periodontitis and no malodor (Table 7). These data indicated that no statistically significant ($P = 0.29$) associations were found between oral malodor and periodontitis in either the whole study sample (n = 127) or the subjects recruited (n = 26) outside of the oral malodor clinic.

DISCUSSION

Relationship of Periodontal Disease to Oral Malodor

Previous studies have indicated that oral malodor often accompanies periodontal diseases.[9–12] Higher VSC levels have been found in subjects with probing depths >4 mm than in subjects with healthy periodontium[11] and the intensity of the odor increases with the severity of the disease.[10-12] Substantial evidence demonstrates that oral malodor may also accelerate the rate of progression of periodontal diseases.[4,6] We have assessed this relationship quantitatively. The results of the study indicated that moderate to severe malodor was detected organoleptically in individuals with both periodontal health and disease. Further, VSC measurements were in good agreement with whole mouth organoleptic measurements which supported this finding. Although the mean VSC level of healthy subjects was not as high as the group with periodontitis, the difference was less than 10% and was not statistically significant ($P > 0.05$). This small difference in recorded VSC levels between the two groups could be attributed to the reduced sensitivity of the sulphide monitor in detecting the elevated amounts of methyl mercaptan produced in periodontitis.[6,11] Mercaptans, which are organoleptically more objectionable than hydrogen sulphide, are detected at approximately 50% of corresponding levels of hydrogen sulphide yet are important contributors to oral malodor. Hydrogen sulphide, a compound formed in both periodontally healthy and diseased mouths,[6,17] is measured much more efficiently by the sulphide monitor (unpublished data). Since significant positive correlations have been established between organoleptic measures and instrumental measures of total oral VSC,[15]

Table 7. Malodor and Periodontal Parameters for Groups With and Without Periodontitis

Variable	Periodontitis/ Halitosis	No Periodontitis/ Halitosis	Periodontitis/ No Halitosis	No Periodontitis/ No Halitosis
Age (years)	35.0± 2.9	51.0±1.9	49.3±3.7	38.3±1.6
VSC Peak (ppb)	126.7±18.8	120.4±6.3	55.7±8.1	71.9±3.3
VSCSS (ppb)	46.7± 8.3	83.3±3.2	29.3±3.5	39.2±2.6
Oral score	3.7± 0.2	3.7±0.1	1.0±0.2	1.2±0.1
Tongue odor	2.3± 0.2	3.3±0.1	1.3±0.2	1.4±0.1
Tongue pH	7.7	6.9	6.9	6.9
Pockets ≥5 mm	2.7± 0.6	0.0	2.3±0.4	0.0
Pockets ≥7 mm	0.3± 0.2	0.0	0.3±0.3	0.0
Gingival index	1.3± 0.1	1.5±0.08	1.3±0.1	1.2±0.1
Plaque index	2.1± 0.1	1.7±0.07	1.5±0.1	1.6±0.1

Measurememts of malodor and periodontitis (mean and standard error) for each of the 4 groups identified in Table 6.

the insignificant (4%) difference in mean organoleptic measurements between the periodontally healthy and diseased groups indicates that an accurate estimate was obtained from the sulphide monitor. Consequently, in contrast to other reports,[7,9,11] we have good reason to believe that both periodontally healthy and diseased individuals can exhibit significant malodor levels and that malodor and VSC are unlikely to be diagnostic of the presence of periodontitis.

Although periodontal pockets are believed to be important sites for VSC production,[6,11] an association between the presence of deep pockets and VSC level was not established in this study. The positive associations reported by other studies may be attributed to the direct, within pocket sampling methods that were used to measure VSC composition and levels[6,11] and that may not be detectable in whole mouth air sampling. Further, due to the inclusion of large numbers of individuals in this study who were self-reported for halitosis consultation, we cannot rule out the existence of confounding bias, the origins of which are currently unknown.

The amount of supragingival plaque estimated by the plaque index was not associated with VSC levels. Eight to 24 hours of maturation are generally required before plaque deposits produce VCS;[29] as the subjects in this study brushed several times per day, smooth surface deposits were repeatedly disrupted before maturation. Thus, in contrast with our previous study,[18] we were unable to demonstrate the importance of readily accessible supragingival plaque in oral malodor production. Although it is possible that each of the studies assessed very different populations, the inclusion of data from all three appointments for the Pearson correlation analysis in our original study may account, in part, for this difference. Since the possibility existed that subjects might alter their oral hygiene habits and subsequently the levels of oral malodor, only first appointment data were analyzed for associations in the present study. When data from all three appointments in our present study were analyzed together, the associations were similar to those found in our previous study.[18] Further, in agreement with an earlier review indicating that protected plaque in approximal sites produces substantial odors and is associated with overall VSC levels,[4] our observations indicated that oral malodor production in healthy individuals may originate, at least in part, from protected interdental sites.

Relationship of Tongue Dorsum to Oral Malodor

Toothbrushing accompanied by tongue cleaning results in a substantial reduction in VSC levels.[30] However, the removal of plaque from tooth surfaces by brushing has been found to be less then half as effective in reducing oral malodor as tongue brushing alone. This suggests that the tongue appears to be an important source of oral malodor[11,25] in periodontally-healthy subjects. Indeed the results of this study indicated that the tongue dorsum contributed to oral malodor and to the VSC content of whole mouth air. These findings are consistent with other studies[11,25,31] indicating that the tongue dorsum is a principal source of oral malodor. However, in contrast with other studies,[11] no difference was found between mean values of tongue odor in subjects with and without periodontitis. This finding supports the observation described above in which oral malodor is not significantly different between periodontally healthy and diseased individuals and further implicates the tongue as an important site of odor production.[31]

Tonzetich[5] showed that brushing the tongue decreased VSC by approximately 75% and reduced oral malodor to an undetectable level in most cases. In contrast, toothbrushing resulted in less than 25% reduction of hydrogen sulphide and mercaptan. Recent work by Yaegaki and Sanada[11] has shown that the tongue is the major contributor in healthy mouths to oral malodor production; but in subjects with periodontal involvement, both tooth-bound plaque and tongue coating contribute to oral malodor. In this context, the coating of the tongue may be an important factor that accelerates VSC production in subjects with and without periodontitis.[9,32] It has been demonstrated that high VSC levels, rich in methyl mercaptans, are produced on the tongue dorsum in subjects with periodontal diseases and that this differs considerably from the amount and composition of VSC (predominantly hydrogen sulphide) produced in healthy subjects.[11] The lower sample size and different patient population used in the Yaegaki and Sanada[11] study may have produced data that are not comparable to our results.

Relationship of Bacteria to Oral Malodor

Oral Gram-negative microorganisms such as spirochetes, fusiforms, vibrios, veillonella, and some bacteroides theoretically have the capacity to produce oral malodor, presumably through putrefaction[33,34] of sulphur-containing protein substrates such as cysteine, cystine, and methionine.[4,6,9,35] The presence of one or more putative periodontal pathogen such as *T. denticola, P. gingivalis*, and *B. forsythus* was identified by the BANA test on 74.4% of tooth and 92.5% of the tongue dorsum sites in healthy individuals. Since the test has a reported sensitivity of 85%, a specificity of 53%, and an accuracy of 79%,[22] there is strong indication that at least one of these periodontal pathogens was present on the tongue of healthy individuals. Indeed, earlier studies have indicated that bacteria are essential for the production of oral malodor[33,34] and, in particular, *F. nucleatum*[36,37] and strains of *P. gingivalis* are capable of metabolizing cysteine and methionine to form VSC.[31,39] The large surface area and papillary structure of the tongue can retain considerable quantities of food and debris and can support and protect a large bacterial population.[40,41] *P. gingivalis,*[42] *T. denticola,*[43] *A. actinomycetemcomitans,*[43] various motile organisms,[44] and *P. intermedia* are found predominantly in deep pockets,[42] but have also been found in healthy gingival sites and on the tongue dorsum of individuals with and without disease.[42] A significant correlation has been found between the pres-

ence of motile organisms ($P < 0.05$) and *P. intermedia*[43] on the tongue in subjects with periodontitis as opposed to periodontally-healthy subjects. This indicates that the tongue may act as a reservoir for some periodontopathogens that contribute to oral malodor. Thus the diverse microorganisms found on the tongue surface[45] could influence the microbiota of the entire oral cavity.

Optimum activity for the production of oral malodor occurs in anaerobic conditions with low carbohydrate[4] availability and above neutral pH.[34,35] No direct association was found between pH and the presence of oral bacteria in this study but there was a decrease in pH after CHX treatment and this corresponded to a statistically significant decrease in tongue and teeth BANA test scores. Regardless of periodontal status, the most objectionable odor is produced when large numbers of disintegrated epithelial cells covered with bacteria are trapped in plaque and crevices of the tongue dorsum.[4] Although we found no significant relationship between decreased salivary activity and oral malodor, oral dryness and/or abstinence from food and liquid provide optimal conditions for putrefaction[1] on dental and tongue sites.

Antimicrobial Effects on Oral Malodor

We used a one week period of CHX rinsing to probe experimentally the importance of bacteria in oral malodor production. Previous clinical studies[46,47] have shown that CHX rinsing significantly reduces the number of plaque microorganisms. Large and significant reductions in VSC levels, organoleptic scores, and positive BANA test scores were measured following a one week rinse with CHX but only small reductions in gingival inflammation and no change in the numbers of deep pockets were noted. These data indicate that the reduction of the microbial load in the oral cavity is important for the control of oral malodor and that, consequently, the growth of bacteria appears to be critical for oral malodor production. However, we were unable to determine quantitatively which oral sites exhibited the largest reduction in bacteria although it appears that dental sites are probably reduced as much as other non-dental sites. For example, the large and contemporaneous reductions of floss odor, VSC, organoleptic, and approximal BANA test scores suggest that oral malodor production from these sites is an important contributor to whole mouth odor.

Our data suggest that dental and tongue sites may be colonized by putative pathogens without evidence of previous periodontal disease. The existence of a carrier state[48] and a prolonged latency period before pathogens initiate overt periodontitis suggests the possibility that the presence of periodontal pathogens in moderate numbers may create oral malodor prior to the initiation of active periodontitis.

CONCLUSIONS

1. Some subjects with periodontitis exhibited high levels of oral malodor but overall these levels were not significantly different from subjects with healthy periodontium. Therefore, oral malodor is not directly associated with the presence of periodontitis; substantial oral malodor may be present in individuals without periodontitis.

2. Organoleptic assessments of the tongue dorsum and approximal plaque were associated with VSC content of whole mouth air and consequently appear to be important sources of oral malodor.

3. Conditions favoring a positive BANA test on the tongue dorsum and at tooth sites were associated with oral malodor production.

4. Rinsing with chlorhexidine resulted in significant reductions of VSC and oral malodor.

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Cadaverine as a Putative Component of Oral Malodor

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Abstract. Whereas previous studies have shown correlations between volatile sulphur compounds (VSC) and bad breath levels, it is probable that other compounds found in the oral cavity may contribute to oral malodor. In the present investigation, the possibility that diamines (cadaverine and putrescine) are associated with oral malodor parameters was assessed. Saliva samples from 52 subjects were analyzed for cadaverine and putrescine by HPLC. Oral malodor of whole mouth, tongue, and saliva of the subjects was recorded by an experienced judge on a continuous 10-cm scale; peak and steady-state VSC intra-oral levels were measured by the Interscan 1170 sulphide monitor. Log-transformed VSC and diamine levels were compared with odor judge measurements by Pearson analysis and stepwise forward multiple regression. Putrescine scores were not significantly associated with odor judge parameters or with VSC levels ($p > 0.1$). However, highly significant correlations ($p \leq 0.003$) were found between cadaverine levels and all three odor judge assessments. In contrast, associations between cadaverine and VSC measurements were non-significant. In an attempt to correlate odor judge results in terms of both VSC and diamines, we carried out stepwise forward multiple regression. Results showed that VSC and cadaverine both factor significantly in explaining each of the odor judge measurements, with multiple r values ranging from 0.545 ($p = 0.0002$) to 0.604 ($p < 0.0001$). The results suggest that cadaverine levels are associated with oral malodor, and that this association may be independent of VSC.

Key words. Diagnostic Tests, Halitosis, Ornithine Decarboxylase, Periodontal Diseases, Saliva.

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Introduction

Oral malodor is a common problem affecting most of the adult population (Tonzetich, 1973, 1977). In the large majority of cases, the origin of bad breath is the oral cavity itself, resulting from microbial putrefaction of food residues and host components. Patients with degenerative periodontal diseases tend to have increased numbers of leukocytes, exfoliated cells, and bacteria in their mouths, as well as higher malodor levels (Tonzetich and Kestenbaum, 1969; Tonzetich, 1973; Kostelc *et al.*, 1981). Most previous investigations have concentrated on the relationship between volatile sulphur components (VSC) and oral malodor (Tonzetich and Richter, 1964; Tonzetich, 1971). We have recently shown that VSC levels, recorded by a portable sulphide monitor, show significant correlations with bad breath, as measured by judges (Rosenberg *et al.*, 1991a,b).

In the present study, we have examined the possibility that cadaverine and putrescine, two malodorous diamines associated with bacterial putrefaction, are associated with bad breath. Regression analyses were carried out to compare the associations of cadaverine and putrescine with malodor and dental-related parameters in a clinical study comprised of 52 subjects.

Materials and methods

Patient population

The study consisted of 52 patients (mean age, 37.9 ± 11.9 years; 35 females), ranging from 11 to 63 years. Among the subjects, 43 complained of bad breath, having responded to a newspaper article on the subject. Exclusion criteria included smokers, subjects suffering from systemic disease

or taking antibiotics, and denture wearers. The study included parameters not reported here, including tests of self-estimation of oral malodor, and psychological data (in preparation).

On the day of the examination, the participants were instructed to refrain from using scented personal products. Subjects were also requested to refrain from oral activities, including drinking, eating, chewing gum, and mouthrinsing, two hours prior to appoinment. The appointment for each subject was randomly scheduled on one of three days during a 12-day period.

Clinical examination

All the participants were scored for plaque accumulation and gingival health of all tooth surfaces (including wisdom teeth) by the Plaque Index (PI; Silness and Löe, 1964) and Gingival Index (GI; Löe and Silness, 1963). Probing depth measurements were recorded for the 30 subjects who were examined on days 2 and 3 of the study (N = 30) with a Michigan O probe, at six reference loci on the buccal and lingual sides of each tooth, including wisdom teeth. All participants were subjected to whole-mouth probing in order to identify shallow and deep pockets for sampling of plaque for the BANA test.

Volatile sulphide concentrations

VSC measurements were made by means of a portable industrial sulphide monitor (model 1170, 1.0 ppm full-scale, Interscan Corp., Chatsworth, CA), zeroed on ambient air before each measurement. A disposable 6.5-mm-circumference plastic straw was inserted into the air inlet of the monitor. Patients were instructed to bring their slightly opened mouths over the straw so that it extended approximately 4 cm into the oral cavity , and to breathe through their noses during the measurement. Both peak (maximum) and steady-state levels attained were determined in parts *per* billion (ppb) sulphide equivalents.

Odor judge assessment

Organoleptic assessment was made by an odor judge with extensive experience (M.R.), who was blinded from all other parameters. Odor judge malodor scores were recorded on a continuous unmarked 10-cm scale, marked at both ends as no odor and maximal odor, respectively. Results were scored independently for (i) whole mouth malodor, (ii) tongue malodor, and (iii) saliva malodor. For measurement of whole mouth malodor, subjects were requested to breathe out from the mouth, at *ca.* 10 cm from the nose of the judge. For measurement of tongue malodor, the subjects were instructed to extend their tongues and lick their wrists in a perpendicular fashion. Five seconds later, the odor judge assessed the odor from a distance of *ca.* 3 cm. For measurement of saliva malodor, the subjects were asked to expectorate into Petri dishes, which were incubated for approx. 5 min at 37°C; the dishes were then assessed by the odor judge at a distance of *ca.* 4 cm. Expectoration was carried out post-probing in order to potentiate saliva odor levels. The same saliva samples were then used immediately for BANA testing, and 100-µL samples were frozen at -20°C for subsequent diamine analysis (below).

BANA assay

The BANA reagent card (Perioscan™, Oral-B Laboratories, Redwood City, CA; Loesche *et al.*, 1990a,b) was used according to manufacturer's instructions. Results were recorded as either strong, dark blue spots (score = 2), weak, light blue spots (score = 1), or no color change (score = 0). For each subject, the average score was calculated for four samples, taken from a deep pocket, a shallow pocket, tongue dorsum, and saliva, in the following manner: Subgingival plaque was removed by curette from teeth presenting a shallow pocket (< 4 mm) and a deep pocket (≥ 4 mm), following local supragingival plaque removal. Subgingival plaque samples were then deposited directly onto the reagent card. A disposable plastic blade was used to obtain tongue samples by scraping the posterior tongue dorsum, and scrapings were applied directly onto the reagent card. Samples (*ca.* 10 µL) of expectorated saliva, taken following probing, were similarly applied onto the reagent card. Results comparing the BANA tests with dental and oral malodor parameters have been described separately (Kozlovsky *et al.*, 1994).

Diamine analysis

For high-performance liquid chromatography (HPLC) analysis, saliva samples were first derived by use of O-phthaldialdehyde reagent. O-phthaldialdehyde reagent solution was prepared by dissolution of 50 µg of O-phthaldialdehyde in 4.5 mL methanol, together with 0.5 mL borate buffer (0.9 M, pH = 9.5) and 50 µL of ß-mercaptoethanol. The solution was diluted 1:10 in methanol and kept at -20°C. To saliva samples (20 µL) were added 80 mL of borate buffer and 25 µL of the diluted reagent solution. The mixture was stirred briefly and injected (80 µL) into the HPLC system. The mobile phase of the HPLC was composed of a filtered, de-gassed 30:70 mixture of 12.5 mM sodium phosphate buffer (pH = 7.2)/acetonitrile. The HPLC system consisted of an Eldex 9600 pump (Eldex Laboratories, Inc., CA), an injection valve equipped with a 20-µL sample loop, and a C18 reverse-phase column (250 x 4 mm Lichrosphere 100 PR-18, 5 µm, Merck, Darmstadt, Germany). Samples were injected at a flow rate of 1 mL/min. Detection was carried out by a UV detector (Spectro Monitor 3,200, LDC Analytical, FL) at a wavelength of 231 nm.

Statistical analyses

Pearson correlation coefficients were used to determine the level of association of the various parameters. Stepwise forward multiple-regression analysis was carried out as

Table 1. Pearson correlation coefficients comparing diamine levels with malodor and dental parameters

	Log [cadaverine]	Log [putrescine]
Malodor Parameters		
Log Peak VSC	r = 0.137	0.112
	p = *1.0*[a]	*1.0*
Log Steady-state VSC	0.057	-0.007
	1.0	*1.0*
Whole mouth (odor judge)	0.375	-0.015
	0.027	
Tongue (odor judge)	0.453	0.127
	<0.009	*1.0*
Saliva (odor judge)	0.477	0.073
	<0.009	*1.0*
Dental Parameters		
PI	0.596	0.066
	<0.009	*1.0*
GI	0.568	0.031
	<0.009	*1.0*
Mean Probing Depth	0.471	0.109
	0.036	*1.0*
Mean BANA Score	0.409	0.006
	0.009	*1.0*

[a] Original p values have been multiplied nine-fold to adjust for multiple comparisons.

indicated. Statistical analyses were performed by SPSS software (SPSS Inc., Chicago, IL).

Results

Cadaverine levels in the 52 saliva samples tested ranged from 0 to 1395.3 μM, with a mean of 371.6 ± 307.6 μM (SD). Putrescine concentrations averaged 109 ± 92.7 μM, ranging from 21.4 to 599.4 μM. Putrescine and cadaverine levels, as well as VSC measurements, were log-transformed in order to obtain near normal distributions.

Pearson correlation coefficients comparing diamine levels with malodor and dental parameters are presented in Table 1. Log cadaverine scores were most highly associated ($p < 0.009$) with odor judge measurement of tongue and saliva, as well as with PI and GI scores. Significant correlations were also observed between cadaverine and the BANA test ($p = 0.009$), whole mouth odor judge scores ($p = 0.027$), and mean probing depth ($p = 0.036$). In contrast, no significant associations were found between cadaverine and VSC levels ($p = 1.0$).

Although log cadaverine and log putrescine scores were significantly associated with one another ($r = 0.482$; $p < 0.001$), log putrescine levels, *per se*, were not found to be significantly associated with any of the malodor or dental parameters (Table 1).

In order to relate each of the three odor judge measurements to both VSC levels and diamine concentrations, stepwise forward multiple-regression analysis was carried out (Table 2). In all three cases (whole mouth, tongue, and saliva), both VSC and log cadaverine levels factored into the regression equation, yielding multiple r values ranging from 0.545 (tongue; $p = 0.0002$) to 0.604 (saliva; $p < 0.0001$). In the case of whole mouth and tongue odor, log steady-state VSC values were not significant following entry of log peak VSC, whereas in the case of saliva, log steady-state levels were entered, and log peak VSC was subsequently left out of the regression equation. Putrescine levels were not significant in any of the analyses.

Stepwise forward multiple-regression analysis of dental indices (PI and GI) in terms of log cadaverine and log putrescine scores is summarized in Table 3. Both PI and GI were best explained by equations combining a positive association with log cadaverine scores and a negative association with log putrescine levels, yielding highly significant ($p < 0.0001$) multiple r values of 0.648 and 0.632, respectively.

Table 2. Stepwise forward multiple-regression analysis of odor judge assessments in terms of VSC and diamine concentrations

		Coefficient for				
Dependent Variable	Intercept	Log Peak VSC	Log Steady-state VSC	Log [cadaverine]	Log [putrescine]	Multiple r
Whole mouth	-9.38	5.86	N.E.[a]	1.55	N.E.	0.556
	0.0029[b]	*0.0009*	*0.3576*	*0.0180*	*0.0513*	*0.0001*
Tongue	-5.43	2.82	N.E.	1.30	N.E.	0.545
	0.0066	*0.0101*	*0.5099*	*0.0023*	*0.4345*	*0.0002*
Saliva	-14.38	N.E.	6.91	2.86	N.E.	0.604
	0.0002	*0.2104*	*0.0015*	*0.0004*	*0.1890*	*<0.0001*

[a] Not entered into the equation due to lack of significance.

[b] Numbers in italics represent levels of significance.

Table 3. Stepwise forward multiple-regression analysis of dental parameters in terms of diamine concentrations

Dependent		Coefficient for		
Variable	Intercept	Log [cadaverine]	Log [putrescine]	Multiple r
Plaque Index	-0.34	0.99	-0.49	0.648
	0.4114[a]	*<0.0001*	*0.0239*	*<0.0001*
Gingival Index	0.00	0.80	-0.44	0.632
	0.9973	*<0.0001*	*0.0155*	*<0.0001*

[a] Numbers in italics represent levels of significance.

Discussion

The present investigation presents initial evidence in support of a role for cadaverine as a component of oral malodor. Cadaverine and putrescine are common bacterial degradation products, and may be produced in saliva as the result of decarboxylation [*i.e.*, of lysine and ornithine (Hayes and Hyatt, 1974)], or, in the case of putrescine, by transamination. Log-transformed cadaverine levels were significantly associated with whole mouth odor ($p < 0.027$), tongue odor ($p < 0.009$), and saliva odor ($p < 0.009$). No significant associations were found when comparing cadaverine with VSC measurements. Multiple-regression analysis indicated that both VSC and cadaverine levels factored significantly in accounting for odor judge measurements. In contrast, no association was found between putrescine and any malodor-associated parameters. The data raise the possibility that cadaverine elaboration in the oral cavity is carried out independent of VSC production, with both factors contributing to the overall oral malodor. This premise is further supported by preliminary *in vitro* data (not shown) showing that saliva samples, inoculated into growth medium, elaborate cadaverine over time, concomitant with appearance of malodor. Cadaverine has been previously shown to be a component of human dental plaque (Hayes and Hyatt, 1974) and is a malodorous product of bacterial putrefaction of meat and fish. Microbial production of cadaverine has been attributed to decarboxylation of lysine (Gale, 1946).

In contrast to cadaverine, putrescine levels, taken alone, did not correlate with any of the dental or malodor-associated parameters. Multiple-regression analysis of PI and GI in terms of cadaverine and putrescine showed that, whereas cadaverine contributed a highly significant positive association, putrescine contributed a significant negative association. These results raise the possibility that putrescine elaboration is inversely related to a factor of cadaverine production which detracts from cadaverine's overall association with PI and GI.

Most previous investigations which have studied the gaseous components contributing to oral malodor have concentrated on volatile sulphur compounds, primarily hydrogen sulphide and methyl mercaptan (Tonzetich and Richter, 1964). Correlations have been found between odor judge ratings and sulphide levels, by use of both gas chromatography and portable monitor techniques (Schmidt *et al.*, 1978). In one study (Tonzetich *et al.*, 1967), indole, methylamine, and cadaverine were added to bulk saliva, with no subsequent increase in odor or gas chromatographic detection of these components in the headspace. However, the gases contributing to oral malodor may be released into the oral headspace when saliva dries out on the mucosal surfaces (Rosenberg and McCulloch, 1992: I. Kleinberg, personal communication). Furthermore, different loci within the oral cavity elaborate odors with different characteristics, suggesting that a wider variety of gases contribute to oral malodor than was previously assumed. In this context, the highly significant correlations comparing cadaverine levels with PI, GI, probing depth, and tongue malodor suggest that both the periodontium and the tongue dorsum may be sites of cadaverine production. The association found here between cadaverine levels and the BANA test suggests that BANA-hydrolyzing micro-organisms present at those loci may be involved in cadaverine production. Experiments are under way to examine the relationship between cadaverine production by oral microbiota and malodor production at different sites in the oral cavity.

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Kinetics of lactose-reversible coadhesion of *Actinomyces naeslundii* WVU 398A and *Streptococcus oralis* 34 on the surface of hexadecane droplets

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Ellen RP, Veisman H, Buivids IA, Rosenberg M. Kinetics of lactose-reversible coadhesion of Actinomyces naeslundii *WVU 398A and* Streptococcus oralis *34 on the surface of hexadecane droplets.*
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Most investigations of mechanisms accounting for intergeneric coaggregation have emphasized stereospecific rather than nonspecific interactions. The purpose of this investigation was to determine the relative importance of lectin-carbohydrate and nonspecific hydrophobic and ionic interactions, using a model based on strains with one of the most well understood specific coaggregation mechanisms, the lactose-reversible coaggregation of *Actinomyces naeslundii* and *Streptococcus oralis*. The kinetics of coadhesion and desorption of coadherent bacteria were studied using *S. oralis* 34 bound to hexadecane droplets as an affinity support for the adhesion of *A. naeslundii* WVU 398A. Light, confocal microscopy and transmission electron microscopy confirmed that *A. naeslundii* cells adhered only to the *S. oralis* cells, not to exposed hexadecane between the streptococci. Coadhesion was inhibited by lactose concentrations as low as 2.0 mM. The rate of coadhesion was halved at 60 mM lactose. The hydrophobicity inhibitors bovine serum albumin and defatted bovine serum albumin and the salts LiCl and KCl failed to inhibit coadhesion in the hexadecane assay, and bovine serum albumin also failed to inhibit coaggregation in a bacterial aggregation assay on glass slides. High concentrations of the salts achieved a 50% rate decrease in *A. naeslundii* adhesion to the *S. oralis*-coated droplets only when they were combined with >20 mM lactose. Sodium dodecyl sulfate (SDS) and Tween 20 inhibition was tested by the slide coaggregation assay because they tended to emulsify the droplets; SDS was inhibitory. Lactose selectively desorbed *A. naeslundii* from *S. oralis*-coated droplets at low concentrations equivalent to those that inhibited coadhesion. Neither LiCl nor KCl desorbed *A. naeslundii* from the droplets, even at 500 mM. At low concentrations, SDS but not Tween 20 eluted both *A. naeslundii* and *S. oralis* from the droplets. Although the SDS results might suggest a degree of cooperative charge interactions, the results support the hypothesis that stereospecific, β-galactoside-sensitive interactions have a much greater impact than nonspecific interactions on the coadhesion of *A. naeslundii* and *S. oralis*.

Key words: coadhesion; kinetics; specificity; hydrophobicity; *Actinomyces naeslundii*; *Streptococcus oralis*

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Coadhesion of dissimilar species influences the sequential establishment of bacteria in dental plaque and helps foster their retention as a biofilm on teeth (6). Experimentally, it is most often studied *in vitro* by mixing 2 bacterial suspensions in the absence of a supporting surface and observing the degree to which they coaggregate during a timed assay. Our laboratory and others have developed *in vitro* assays in which one species is added to cells of another which have first been adsorbed as a monolayer on a surface (1, 8, 11, 15, 16, 18). Such assays allow investigations of the rate of coadhesion and the effect of inhibitors on the kinetics of coadhesion. They are particularly useful for simulat-

ing the order in which the selected species usually re-establish on cleaned teeth and for conducting experiments when conventional coaggregation assays are difficult to assess because one or both of the paired species self-aggregates (9, 10, 16).

Studies of coaggregation mechanisms have most often emphasized the stereospecificity of interacting molecules on the surfaces of the pairs. Most pairs of oral bacteria that coaggregate have been reported to interact by lectin-carbohydrate recognition (6,7). With few exceptions (13, 15), neither the avidity of coaggregation nor the potential contribution of nonspecific interactions to the stability of developing coaggregates has been addressed quantitatively. We have recently introduced an assay by which the rate of coadhesion on the surfaces of hexadecane droplets and the influence of inhibitors of hydrophobicity can be measured. Its utility in studying the kinetics of *Porphyromonas gingivalis-Actinomyces viscosus* interactions and in documenting the effects of proteases and other proteins on coadhesion has been demonstrated (5, 15). However, using it to determine the relative importance of specific versus nonspecific interactions is currently premature with these 2 species, because specific mechanisms underlying their mutual recognition have not been well characterized at the molecular level.

The most well documented specific coaggregation interaction among oral bacteria is the β-galactoside-inhibitable reaction between *Actinomyces naeslundii* and oral streptococci such as *Streptococcus oralis* (6, 19). During plaque formation *in vivo*, the adsorption of *S. oralis* to salivary pellicle on teeth or simulations of tooth surfaces typically precedes that of *A. naeslundii* (3, 12). Therefore, we selected a pair of well characterized, coaggregating strains to study the relative significance of stereospecific and electrostatic or hydrophobic interactions in establishing and disrupting their coadhesion. The test hypothesis was that galactoside-inhibitable and -reversible interactions have a far greater impact than nonspecific forces on the kinetics of *A. naeslundii-S. oralis* coadhesion.

Material and methods

Bacterial strains and culture conditions

A. naeslundii WVU 398A was originally obtained from M. A. Gerencser, West Virginia University, and maintained in the lyophilized culture collection at the University of Toronto. Stocks were maintained at 4°C on brain heart infusion agar slants (Difco Laboratories, Detroit, MI). For experiments, 10-ml tryptic soy broth (Difco Laboratories) cultures were grown for 48 h from inocula off the slants, and these were used at a 1 : 100 dilution to inoculate broth culture tubes for a subsequent 48-h growth period in an atmosphere of 80% N_2, 10% CO_2 and 10% H_2. Cells were harvested by refrigerated centrifugation and washed twice in modified coaggregation buffer (7) containing 121.1 mg TRISMA-Base ((Sigma Chemical Co., St. Louis, MO) 14.7 mg $CaCl_2 \cdot H_2O$, 20.3 mg $MgCl \cdot 6H_2O$, 8.77 g NaCl, per liter distilled H_2O, pH 8.0). Coaggregation buffer suspensions were prepared in glass test tubes at various optical densities that were corrected for nonlinearity, ranging downward from 1.2 at 550 nm (OD_{550}) in a model 350 spectrophotometer (G. K. Turner Associates, Palo Alto, CA). The optimum OD_{550} for coadhesion assays was found to be 1.0 and for slide coaggregation assays 1.2.

S. oralis 34 was originally obtained from R. J. Gibbons, Forsyth Dental Center and maintained lyophilized. Stock cultures were maintained on tryptic soy agar plates containing 5% defibrinated sheep blood. For experiments, *S. oralis* was grown aerobically in tryptic soy broth for 24 h and then inoculated at 1 : 100 dilution for an additional 24 h growth. Cells were washed and suspended in coaggregation buffer as described above.

Adhesion to hexadecane

To confirm their innate hydrophobicity, the adhesion of *A. naeslundii* 398A and *S. oralis* 34 to hexadecane was measured prior to their use in coadhesion assays (15). Cell suspensions ($OD_{550} = 1.2$) were distributed in 2.0-ml aliquots into borosilicate glass tubes; 0.15 ml *n*-hexadecane was added to each tube. The suspensions were mixed at the highest setting on a Vortex-Genie (Scientific Industries, Bohemia, NY) for 4 consecutive 1-min periods. Following phase separation, the change in turbidity of the aqueous phase was measured. For *S. oralis* 34, the contents of the test tubes were pooled, and the aqueous phase removed, yielding hexadecane droplets coated with streptococci. The optimum OD_{550} to produce *S. oralis* 34-coated hexadecane droplets for coadhesion experiments was found to be 1.0. The *S. oralis*-coated droplets were washed by gently swirling them with fresh coaggregation buffer as the aqueous phase to ensure their uniform distribution prior to dispensing into test tubes.

Coadhesion and coaggregation assays

Coadhesion of *S. oralis*-coated hexadecane droplets and *A. naeslundii* 398A followed the methods for *P. gingivalis-A. viscosus* pairs that we had published previously (15). Briefly, to 1.0 ml of *A. naeslundii* suspension, OD_{550} 1.0, was added 0.3 ml of coaggregation buffer, 0.2 ml of inhibitor and 0.5 ml of *S. oralis*-coated droplets. When a second inhibitor was added, volumes of buffer and inhibitor were varied accordingly to maintain a final volume of 2.0 ml. Controls included: 1) tubes containing *n*-hexadecane free of *S. oralis*; 2) tubes containing only *S. oralis*-coated droplets in buffer, with or without inhibitors, and 3) tubes containing *S. oralis*-coated droplets and *A. naeslundii* as a positive control when the effect of inhibitors was determined. All tubes were incubated at ambient temperature with gentle rotation at a fixed 40° incline in a multipurpose rotator (Scientific Industries). At 2-min intervals, phases were allowed to separate, and the turbidity of the lower phase was measured directly.

Three microscopy methods were used to examine the *S. oralis*-coated droplets, with and without adherent *A. naeslundii* cells. 1) For conventional light microscopy, the droplets were exposed to 1.0 ml of aqueous solution of safranin diluted 1/10, allowed to rotate for 15 min and washed 5 times in coadhesion buffer. *A. naeslundii* 398A cells were suspended for 5 min in 1.0 ml of crystal violet diluted 1/100 and washed 7 times with buffer. The lower aqueous phase in the tubes containing the safranin-stained *S. oralis* droplets was replaced with 2.0 ml of a 1 : 1 mixture of the *A. naeslundii* suspension and buffer, and the droplets were rotated for 1 h. 2) To stain the streptococci for confocal microscopy, the *S. oralis*-coated droplets were rotated for 30 min with 10 μg/ml acridine orange in the aqueous phase and washed 3 times. They were then rotated for 1 h with the usual *A. naeslundii* suspension in coadhesion buffer until the aqueous phase was no longer turbid and washed again. The bound *A. naes-*

lundii cells were stained by indirect immunofluorescence using a 1/100 dilution of rabbit anti-*A. naeslundii* 398A whole-cell antiserum followed by 5 μg/ml rhodamine-conjugated anti-rabbit globulin, with intervening wash cycles. The samples were scanned with a Leica Fluovert Confocal Laser Scanning Microscope (Leica Lasertechnik, Heidelberg, Germany) using dual channel detection: one channel with an OG590 filter to detect rhodamine emissions and the other with an OG515 filter to detect acridine orange emissions. Twenty 2.0-μm optical sections in the x/y plane, scanned 4 times each, were reconstructed into a single image by an extended focus reconstruction program, and stereo pairs were created optically. 3) Samples of *S. oralis*-coated droplets and droplets with bound *A. naeslundii* were also fixed and embedded for conventional ultrathin sectioning and transmission electron microscopy.

The degree of coadhesion was calculated as the percent decrease in turbidity of the aqueous phase when compared with the initial reading. The rate of coadhesion was also calculated from the slopes of log [(at/a0) × 100] versus time, where "at" is the absorbance$_{550}$ for time "t" and "a0" is the absorbance$_{550}$ for time "0". These data were used to generate regression curves for rate in response to inhibitor concentration. Experiments were repeated at least twice; representative data are illustrated.

Inhibitors considered nonspecific included the detergents Tween 20 and sodium dodecyl sulfate (SDS), the hydrophobicity-inhibiting proteins bovine serum albumin (BSA) and defatted BSA and the salts LiCl and KCl, which would be expected to affect both electrostatic and hydrophobic interactions. The coadhesion assay was also tested over a pH range from pH 5.5 to pH 10. Lactose was selected as the specific inhibitor because it is a soluble β-galactoside used routinely to inhibit and reverse streptococcal – *A. naeslundii* coaggregation (7, 13). Its inhibitory concentrations were compared with that of β-methyl and α-methyl galactoside, with glucose included as a control. All reactions were done in the presence of calcium, as it is required for the lactose-sensitive lectin-like functions of *A. naeslundii* (4). The inhibitors were added singly or in combination to the mixture over a wide range of concentrations to determine the effect on coadhesion rate, and they were also added to *S. oralis*-coated droplets with preattached *A. naeslundii* cells to determine their effect on the *A. naeslundii* desorption rate. For assays combining the inhibitors, the lactose concentration was adjusted above and below 60 mM (the concentration closest to halving the coadhesion rate [when coadhesion rate data were expressed by simple regression curve] while achieving 100% inhibition) and combined with a broad range of concentrations for the salts.

Because Tween 20 and SDS emulsified the hexadecane droplets over the concentration range used, inhibition experiments with these detergents and BSA were conducted by coaggregation on glass slides, as reported previously (9). One drop each of *S. oralis* 34 and *A. naeslundii* 398A suspensions were mixed in wells of a flocculation slide and agitated at ambient temperature on a TekTator V serological rotator (American Hospital Supply Corp., Evanston, IL) at a speed of 90 rpm for 5 min. The degree of coaggregation was scored visually, with 0 representing no visible aggregation and 4+ assigned to aggregates yielding a single large clump. BSA and defatted BSA were tested at final concentrations ranging from 0.33 mg/ml to 1.67 mg/ml. SDS was tested at final concentrations ranging from 0.017% to 0.33% (wt/vol) and Tween 20 from 0.017% to 0.33% (vol/vol).

Desorption of *A. naeslundii* from *S. oralis*-coated hexadecane droplets

S. oralis-coated droplets with adsorbed *A. naeslundii* cells were washed by replacing the *A. naeslundii* suspension remaining in the aqueous phase with fresh, bacteria-free coaggregation buffer and then replacing it again with new buffer containing various concentrations of the inhibitors or control solutions. Lactose and glucose were tested over a concentration range of 10–100 mM. LiCl and KCl were tested over a range of 100–500 mM. For experiments using a combination of lactose and one of the salts, the lactose concentration was held constant at 60 mM. Tween 20 and SDS were also tested by incorporating them in the aqueous phase at concentrations 0.04–0.75% (wt/vol and vol/vol respectively) and gently rotating the tubes. The rate of desorption of bacteria was determined by measuring the increase in turbidity of the aqueous phase with time. Samples of the bacteria in the aqueous phase were stained and examined by light microscopy to determine whether *A. naeslundii* alone or both species desorbed.

Results

Coadhesion experiments

Both *A. naeslundii* 398A and *S. oralis* 34 adhered readily to *n*-hexadecane, in-

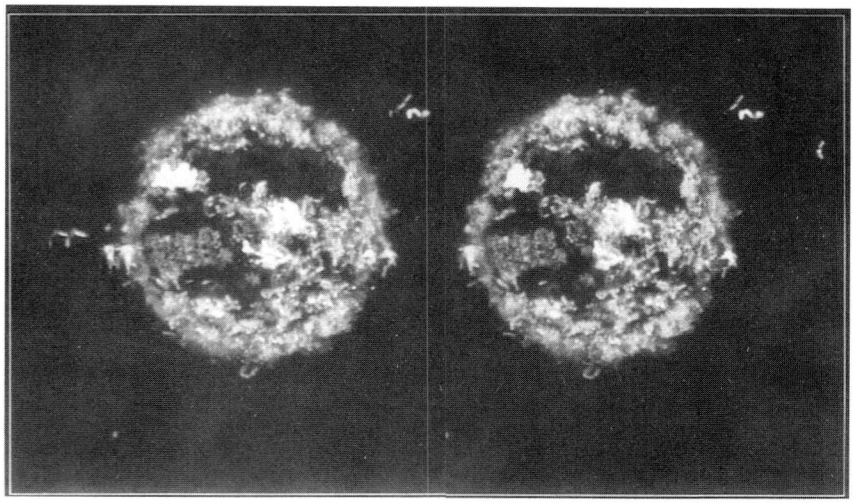

Fig. 1. Stereo pair of reconstructed confocal microscopy image of a *Streptococcus oralis* 34-coated hexadecane droplet following coadhesion assay with *Actinomyces naeslundii* WVU 398A. Adherent streptococci appear as small, light gray round cells, bound directly to the droplet surface. *A. naeslundii* cells, seen as brightly fluorescent rod-shaped bacteria due to adsorbed rhodamine-conjugated second antibody, adhered only to the streptococci, not to the bacteria-free hexadecane. The distribution of the bound bacteria is best seen in 3 dimensions by using a stereo viewer centered over the figure.

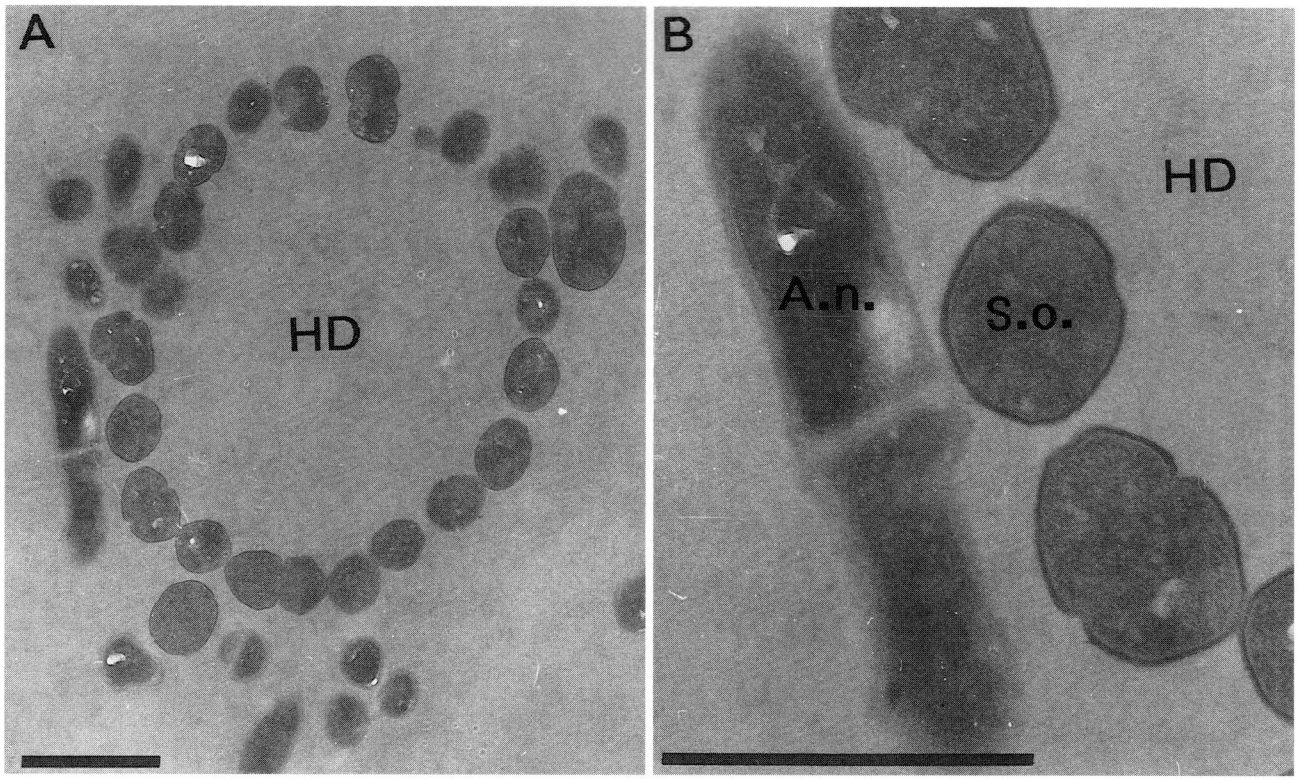

Fig. 2. Transmission electron photomicrographs of ultrathin sectioned *A. naeslundii* (A.n.) adhering to *S. oralis* (S.o.)-coated hexadecane (HD) droplet, demonstrating **A)** a section through an intact droplet and **B)** the close association of the two species connected by an electron-dense material. Bar = 1.0 μm.

dicating that their surfaces contain hydrophobic moieties. *S. oralis*-coated hexadecane droplets remained stable for days at ambient temperature, except when mixed with detergents. Light microscopy of droplets revealed areas containing monolayers of contiguous *S. oralis* cells separated by areas of hexadecane free of bacteria. When examined by both light and laser confocal microscopy, differential staining confirmed that the *A. naeslundii* cells bound only to the *S. oralis* cells, not to the bacteria-free areas (Fig. 1). The microscopy observations were further supported by turbidity measurements for the control tubes that contained hexadecane but no *S. oralis*-coated droplets. The turbidity of the aqueous phase remained constant throughout the assay, indicating that few, if any, *A. naeslundii* cells bound to hexadecane at the interface while the tubes were rotated gently. Electron microscopy confirmed that the *A. naeslundii* cells bound directly to *S. oralis* via dense surface coatings of both species and that neither species penetrated or deformed the hexadecane droplets (Fig. 2).

The β-galactosides lactose and β-methyl-galactoside inhibited the adhesion of *A. naeslundii* 398A to the *S. oralis*-coated droplets compared with controls containing either no potential inhibitor, α-methyl galactoside or glucose (Fig. 3). The minimum lactose concentration yielding any inhibition in this assay was 2.0 mM. Fig. 4 illustrates the effect of lactose on coadhesion rate over a wide range of concentrations. The data could be expressed as either a single straight line, 2 lines of different slope or a sigmoidal curve, all with high R^2 values. A minimum final concentration of 60 mM lactose was sufficient to decrease the rate by approximately half yet maintain a level of inhibition close to 100% (Fig. 3, 4).

The potential inhibitors of hydrophobic interactions that could be used in the hexadecane droplet assay, BSA

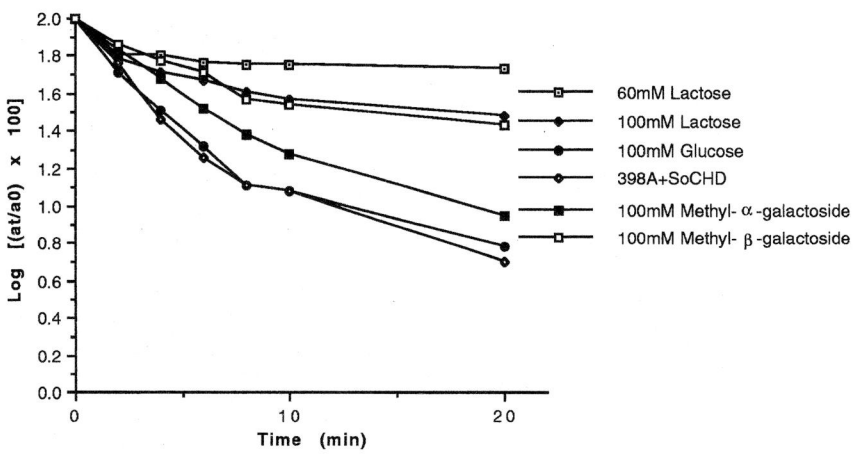

Fig. 3. The effect of galactosides and glucose control on the adhesion of *A. naeslundii* to *S. oralis*-coated hexadecane droplets. Data are expressed as the log percentage decrease in turbidity as a function of mixing time, where "a*t*" is the absorbance for time *t* and "a0"is the absorbance for time zero, just before mixing.

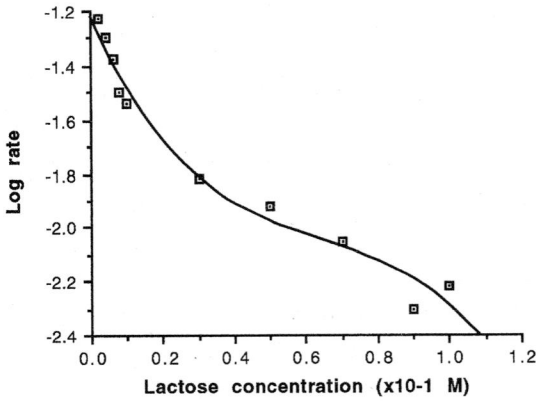

Fig. 4. Curve illustrating the rate of *A. naeslundii* adhesion to *S. oralis*-coated hexadecane droplets as a function of lactose concentration. A sigmoidal curve is shown, as it yielded the highest R^2 value ($R^2 = 0.975$). The data set could also be represented by a single, straight regression curve ($R^2 = 0.931$).

and defatted BSA, not only failed to inhibit coadhesion but actually increased the adhesion of *A. naeslundii* slightly at all concentrations (data not shown). They also failed to inhibit coaggregation in the slide assay (Table 1). SDS, but not Tween 20, was inhibitory in the slide coaggregation assay (Table 1). The salts LiCl and KCl yielded only minor inhibition of adhesion of *A. naeslundii* 398A to *S. oralis*-coated droplets, even at concentrations as high as 500 mM (Table 2). The salts achieved 50% coadhesion rate inhibition only when combined with >20 mM lactose. At lower lactose concentrations, even 500 mM of the salts failed to lower the coadhesion rate by more than 20%. There was no notable difference in coadhesion over a wide range of buffer pH from pH 5.5 to pH 10.

Desorption experiments

Adhesion of *A. naeslundii* to the *S. oralis*-coated droplets was rapidly reversible only when exposed to the specific inhibitor lactose in the aqueous phase (Fig. 5, 6). At concentrations 60 and 100 mM, lactose caused almost immediate desorption, reaching a plateau by 5 min (Fig. 5). Even at a concentration as low as 10 mM lactose, the desorption rate was more rapid than for the glucose control at 100 mM. No desorption was evident when the droplets were rotated with buffer as the aqueous phase. Neither LiCl (Fig. 6) nor KCl (data not shown; identical to LiCl) caused significant desorption of *A. naeslundii* from the *S. oralis*-coated droptlets, even at the highest concentration tested, 500 mM. Moreover, these salts did not noticeably alter the desorption rate for 60 mM lactose.

During the short period of the desorption experiment, some measurements of the effect of low concentrations of Tween 20 and SDS were possible. At the highest final concentration tested, 0.75%, SDS yielded desorption at a rate similar to lactose (Fig. 7). In contrast, Tween 20 had no effect. Microscopic examination of the bacteria desorbed by SDS yielded mixed morphotypes resembling both *A. naeslundii* and *S. oralis*, whereas bacteria desorbed by lactose were virtually all of an *Actinomyces* morphotype. Visual inspection of the separated phases indicated that SDS destabilized the hexadecane droplets.

Discussion

Data from the coadhesion assay used in this investigation support the hypothesis that stereospecific, β-galactoside-sensitive interactions have a greater impact than nonspecific interactions on the adhesion of *A. naeslundii* to *S. oralis*. The selectivity of *A. naeslundii* adhering to *S. oralis* cells rather than directly to hexadecane at the hydrocarbon-aqueous interface might represent the most graphic example of rapid attraction due to the avidity of specific interbacterial recognition mechanisms. Coadhesion was inhibitable and reversible at very low concentrations of lactose. In contrast, even high concentrations of salts known to disrupt hydrophobic interactions and to alter electrostatic interactions were ineffective unless combined with lactose well above its minimum inhibitory concentration. Moreover, coadhesion was not affected over the pH usually encountered *in vivo*. BSA, which is known to prevent hydrophobic and perhaps other nonspecific interactions and is often added to biological assays as a nonspecific blocker, had no inhibitory effect on either adhesion of *A. naeslundii* to *S. oralis* cells bound to hexadecane droplets or coaggregation between cells of the 2 species. Indeed, adding BSA actually increased the number of *A. naeslundii* cells bound.

The results do not fully negate the importance of nonspecific interactions in the surface attraction between *A. naeslundii* and *S. oralis*. Our obser-

Table 1. Slide coaggregation, *A. naeslundii* 398A and *S. oralis* 34

Bacteria	Test agent (final concentration)		Coaggregation score
S. oralis 34	Buffer		0
A. naeslundii 398A	Buffer		0
S. oralis and *A. naeslundii*	Buffer		4+
S. oralis and *A. naeslundii*	BSA	0.33 mg/ml	4+
		0.67 mg/ml	4+
		1.67 mg/ml	4+
	Defatted BSA	0.33 mg/ml	4+
		0.67 mg/ml	4+
		1.67 mg/ml	4+
	SDS	0.017%	2+
		0.003%	2+
		0.067%	1+
		0.167%	0
		0.333%	0
	Tween 20	0.017%	4+
		0.033%	4+
		0.067%	4+
		0.167%	4+
		0.333%	4+

Table 2. The effect of salts on the rate of coadhesion

	Coadhesion rate ($\times 10^{-2}$)[a] Final lactose concentration			
	60 mM	20 mM	2 mM	0 mM
Controls				
	1.49	1.37	2.53	2.95[b]
KCl (final concentration)				
10 mM	1.05	1.14	2.66	2.84
100 mM	0.64	0.91	2.30	2.14
200 mM	0.47	0.84	2.13	2.45
500 mM	0.24	0.94	2.24	2.39
LiCl (final concentration)				
10 mM	1.07	1.32	2.55	2.86
100 mM	0.81	1.05	2.57	2.52
200 mM	0.62	1.09	2.31	2.40
500 mM	0.40	0.74	2.44	2.44

[a] Coadhesion rate is defined as the slope of the curve expressing log $[(at/a0) \times 100)$ as a function of time, where "a*t*" is the absorbance for time "*t*" and "a0" the absorbance for time "0". [b] In different experimental runs this value ranged from 2.95 to 3.04.

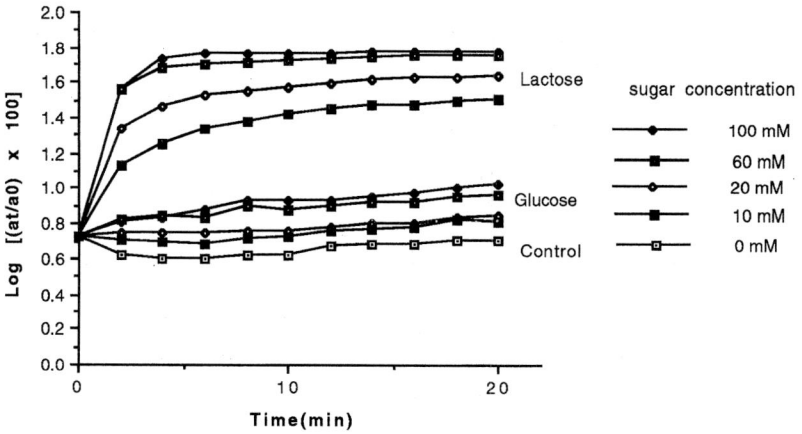

Fig. 5. The effect of various concentrations of lactose and the glucose control on the desorption of *A. naeslundii* from *S. oralis*-coated hexadecane droplets. Data are expressed as the log percentage increase in turbidity as a function of mixing time, where "a*t*" is the absorbance for time *t* and "a0" is the absorbance for time zero, just after replacing the *A. naeslundii* aqueous suspension with fresh bacteria-free buffer.

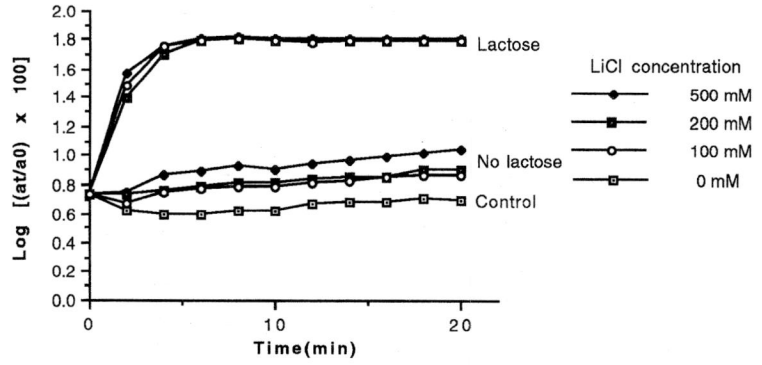

Fig. 6. The effect of various concentrations of LiCl with and without 60 mM lactose on the desorption of *A. naeslundii* from *S. oralis*-coated hexadecane droplets. Data are expressed as in Fig. 5.

vations confirm a previous report by McIntire et al. (13) that such amphipathic agents as SDS, singly and in combination with β-galactosides, reduce the degree of coaggregation between *A. naeslundii* (classified then as *Actinomyces viscosus*) T14V and *S. oralis* (classified then as *Streptococcus sanguis*) 34. While SDS clearly inhibited coaggregation by the slide test, its effect on the kinetics of coadhesion could not be determined because it disrupted the *S. oralis*-coated droplets. Therefore, it is difficult to differentiate whether inhibition derived solely from its interfering with attractive forces between the *S. oralis* and *A. naeslundii* cells. It is conceivable that detergent effects would be more significant in preventing or disrupting the homotypic adhesion between like bacterial cells than the heterotypic coadhesion between cells of different species. Our photomicrographs of droplets show that, whereas the *S. oralis* cells are densely crowded and in contact, the *A. naeslundii* cells do not adsorb closely enough together to establish avid lateral contacts with each other. The more generalized effect of SDS on homotypic adhesion is supported by the microscopic observation that SDS released both *A. naeslundii* and *S. oralis* from the droplets, whereas lactose released bacteria predominantly of an *Actinomyces* morphotype. Thus, results with inhibitors in coadhesion assays using a support surface might better represent the nature of coadhesive interactions important in establishing a sparsely populated biofilm or in selectively attracting a new species to the biofilm from the surrounding fluid suspension. Moreover, interpretation of inhibition results might be clearer in coadhesion than in coaggregation assays, because the former are amenable to analysis of both rate kinetics and endpoint.

Alternatively, the effects of "nonspecific" inhibitors, such as BSA or detergents, might be more specific than expected. In our laboratory's study of *P. gingivalis-A. viscosus* coadhesion, BSA and defatted BSA altered the slope of the coadhesion rate curve, but they did not totally inhibit the reaction (15). Subsequently, we found that several other proteins also prevented coadhesion, and we concluded that BSA was an inhibitor possibly because it served as a specific substrate for cell-associated proteases or other

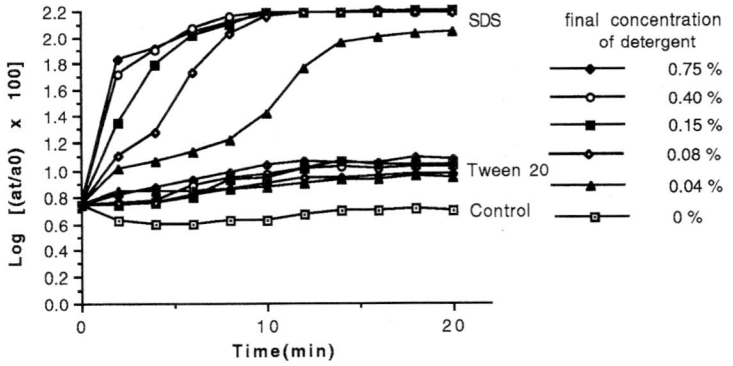

Fig. 7. The effect of various concentrations of SDS and Tween 20 on the desorption of bacteria from *S. oralis*-coated hexadecane droplets. Data are expressed as in Fig. 5.

specific binding molecules on *P. gingivalis* (5). Whether this pertains to *A. naeslundii-S. oralis* interactions remains an open question. Moreover, amphipathic molecules classified as detergents might have other nondetergent biological effects. Both our findings and the McIntire et al. coaggregation inhibition results demonstrate differences for SDS and Tween compounds. Indeed, McIntire et al. concluded that SDS inhibition of *A. viscosus-S. sanguis* coaggregation was not due to an ordinary detergent effect but due to the binding of SDS to the *A. viscosus* lectin in close proximity to the galactoside-combining locus (13). That is, it interfered with a stereospecific interaction.

KCl and LiCl had rather similar but limited inhibitory effects. Very high concentrations of these salts were required to alter the coadhesion rate, and they did not cause desorption. Lower salt concentrations affected coadhesion only in the presence of much lower concentrations of the specific inhibitor lactose. The effect of ionic concentration was much less than that previously reported for the adhesion of *A. viscosus* (20), and the effect of chaotropic LiCl was much less than that reported for the adhesion of *S. sanguis* to saliva-coated hydroxyapatite (14, 20). Our results suggest that the salts might cause a minor charge effect which impacts on β-galactoside inhibition. Despite ample evidence that hydrophobicity is crucial for cooperativity in adhesion of oral streptococci to hydroxyapatite, our data for *S. oralis* 34 coadhesion with *A. naeslundii* 398A do not fully satisfy all the criteria for a multiple class binding mechanism as cited by Doyle et al. (2). Yet curves generated from data expressing the relationship between lactose concentration and rate suggest that specific coadhesion interactions between the bacteria are more complex than if binding interactions between the adhesion molecules themselves were studied in isolation. Nevertheless, it is evident that, in this *in vitro* system, stereospecific interactions dominate coadhesion, supporting recent evidence that lectin specificity may be a significant determinant of the intraoral distribution of *Actinomyces* strains (19) and their establishment on surfaces coated with coaggregation-positive streptococci (17). Although pH, ionic concentration and chaotropic agents should destabilize noncovalent interactions, the multiple lectin-carbohydrate interactions between whole cells of *A. naeslundii* and *S. oralis* must be so avid that conditions way beyond the physiologic range seem necessary to affect coadhesion. It follows that if preventing or disrupting selectively targeted coaggregation reactions in dental plaque is to become an eventual therapeutic goal, then incorporating specific inhibiting or desorbing agents might be advantageous.

Acknowledgements

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Correlation between the BANA Test and Oral Malodor Parameters

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Abstract. The purpose of the present investigation was to test the association between the BANA test (Perioscan®, Oral-B), and oral malodor parameters. The subject population consisted of 52 Israeli adults, 43 of whom complained of oral malodor. Oral malodor measurements consisted of peak and steady-state volatile sulphide measurement by a portable sulphide monitor (Interscan Corp., model 1170), as well as organoleptic measurements of malodor from whole mouth, tongue, and saliva. Samples for the BANA test were obtained from four loci (shallow pocket, deep pocket, tongue dorsum, saliva); results were scored as negative (0), weak (1), or strong (2). BANA scores were significantly associated with odor-judge ratings, with the highest association obtained when BANA saliva scores and odor-judge saliva assessment were compared ($r = 0.500$; $p < 0.001$). BANA tests from the different loci were not significantly associated with sulphide monitor levels. Stepwise multiple-regression analysis of odor-judge measurements in terms of sulphide levels and average BANA scores showed that both log peak sulphide levels as well as BANA scores were significantly factored into the equations, yielding, in all cases, highly significant correlations (multiple $r = 0.57$, 0.50, and 0.59, respectively, with significance levels of 0.0001, 0.001, and < 0.0001, for whole mouth, tongue, and saliva malodor, respectively). The results suggest that the BANA scores are associated with a component of oral malodor which is independent of volatile sulphide measurements and suggest its use as an adjunct test to volatile sulphide measurement.

Key words. Diagnostic Tests, Halitosis, Periodontal Diseases, *Porphyromonas gingivalis*, *Treponema denticola*.

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Introduction

Oral malodor (also known as bad breath, halitosis, *fetor ex ore*) usually originates when the halitus emitted through the mouth combines with malodorous volatile compounds originating in the oral cavity (Tonzetich, 1977). Tonzetich and Richter (1964) were the first to report that oral malodor is primarily associated with volatile sulphur compounds (VSC), rather than with amines and ammonia. VSC are produced in the oral cavity through the putrefactive action of micro-organisms on exogenous and endogenous proteinaceous substrates, including exfoliated cells, leukocytes, saliva, blood, and food debris (Tonzetich and Kestenbaum, 1969; Tonzetich, 1977). The most popular method for instrumental analysis of VSC has been gas chromatography (GC) (Tonzetich, 1971). More recently, a portable sulphide monitor has been proposed for the rapid chairside measurement of overall sulphide levels (Rosenberg *et al.*, 1991a,b). Quantitative measurement is often accompanied by hedonic (organoleptic) measurement by one or more odor judges (Rosenberg and McCulloch, 1992). Both quantitative techniques of gas analysis have been shown to be significantly correlated to hedonic measurements (Schmidt *et al.*, 1978; Rosenberg *et al.*, 1991a,b). Subjects with periodontal disease frequently suffer from oral malodor (Tonzetich, 1973; Tonzetich and Ng, 1976), and positive correlations have been demonstrated between the severity of periodontitis and VSC levels in mouth air (Tonzetich and McBride, 1981; Yaegaki and Sanada, 1992) as well as in pockets (Rizzo, 1967; Coil and Tonzetich, 1985; Mardinger *et al.*, 1992). Many oral bacteria, including those isolated from deep periodontal pockets, are capable of producing VSC, primarily hydrogen sulfide and methyl mercaptan, from serum and from L-cysteine and L-methionine (Persson *et al.*, 1990).

Three of the bacterial species associated with periodontitis—*Porphyromonas gingivalis* (Loesche *et al.*, 1985), *Treponema denticola* (Moore *et al.*, 1985; Simonson *et al.*, 1988), and *Bacteroides forsythus* (Dzink *et al.*, 1988)—are among the most active hydrogen sulphide producers *in vitro* (Persson *et al.*, 1990). The presence of these organisms in dental plaque can be demonstrated based on their ability to hydrolyze the synthetic trypsin substrate N-benzoyl-DL-arginine-2-naphthylamine (BANA; Laughon *et al.*, 1982; Tanner *et al.*, 1985; Loesche, 1986; Loesche *et al.*, 1987).

The purpose of the present investigation was to examine the relationship between the presence of periodontopathic micro-organisms in dental plaque, tongue scrapings, and saliva, as measured by the BANA assay, with instrumental and organoleptic measurements related to oral malodor.

Materials and methods

Patient population

The study consisted of 52 patients (mean age, 37.9 ± 11.9 years; 35 females), ranging from 11 to 63 years old. Among the subjects, 43 complained of bad breath, having responded to a newspaper article on the subject. Exclusion criteria included smokers, subjects suffering from systemic disease or taking antibiotics, and denture wearers. The study included parameters not reported here, including salivary cadaverine levels (Goldberg *et al.*, 1994), as well as tests of self-estimation of oral malodor, and psychological data (in preparation).

On the day of the examination, the participants were instructed to refrain from using scented personal products. Subjects were also requested to refrain from oral activities, including drinking, eating, chewing gum, and mouthrinsing two hours prior to appointment. Measurements were obtained on three dates within over a 12-day period.

Clinical examination

All the participants were scored for plaque accumulation and gingival health of all tooth surfaces (including wisdom teeth) by the Plaque Index (PI; Silness and Löe, 1964) and Gingival Index (GI; Löe and Silness, 1963). Probing depth measurements were recorded on days 2 and 3 of the study (N = 30) with a Michigan O probe, at six reference loci on the buccal and lingual sides of each tooth, including wisdom teeth. All participants were subjected to whole-mouth probing so that shallow and deep pockets could be identified for BANA sampling.

BANA assay

The BANA reagent card (Perioscan®, Oral-B Laboratories, Redwood City, CA; Loesche *et al.*, 1990a,b) was used according to manufacturer's instructions. Results were recorded as either: strong, dark blue spots (score = 2); weak, light blue spots (score = 1); or no color change (score = 0). Subgingival plaque was removed by curette from a tooth presenting a shallow pocket (< 4 mm) and one presenting a deep pocket (≥4 mm), following local supragingival plaque removal. Subgingival plaque samples were then deposited directly onto the reagent card. Tongue samples were obtained by scrapings from the posterior tongue dorsum, obtained by means of a disposable plastic blade, and applied directly onto the reagent card. Saliva samples were taken following the dental examination by asking the subjects to expectorate into a Petri dish. Aliquots (*ca.* 10 µL) of expectorated saliva were immediately applied to the reagent card. The same sample expectorates were subsequently used for odor-judge measurements (below).

Volatile Sulphide Concentrations

VSC measurements were made by use of a portable industrial sulphide monitor (model 1170, 0.5 ppm full-scale, Interscan Corp., Chatsworth, CA), zeroed on ambient air before each measurement. A disposable 6.5-mm-circumference plastic straw was inserted into the air inlet of the monitor. Each patient was instructed to bring his/her slightly opened mouth over the straw so that it extended approximately 4 cm into the oral cavity, and to breathe through the nose during the measurement. Both peak (maximum) and steady-state levels attained were determined in parts *per* billion (ppb) sulphide equivalents.

Odor-judge assessment

Organoleptic assessment was made by an odor judge (M.R.) whose organoleptic scores have been shown to correlate with those of other judges, as well as volatile sulphide levels, in previous studies (Rosenberg *et al.*, 1991a,b). Organoleptic malodor scores were recorded on a continuous unmarked 10-cm scale, marked at both ends as no odor and maximal odor, respectively. Results were scored independently for (i) whole mouth malodor, (ii) tongue malodor, and (iii) saliva malodor. For measurement of whole-mouth malodor, subjects were requested to breathe out from the mouth at *ca.* 10 cm from the nose of the judge. For measurement of tongue malodor, the subjects were instructed to extend their tongue and lick their wrist in a perpendicular fashion. The odor judge assessed the odor from a distance of *ca.* 3 cm, following 5 s. For measurement of saliva malodor, the subjects were asked to expectorate into Petri dishes, which were incubated for approximately 5 min at 37°C, and then assessed at a distance of *ca.* 4 cm from the nose of the odor judge. Expectoration was carried out post-probing in order to potentiate saliva odor levels. VSC and malodor measurements were carried out following the dental examination.

Statistical analyses

Pearson and Spearman correlation coefficients were used as indicated. ANOVA was used to test for differences in log VSC scores over the three days of the study. To test for

Table 1. Frequency distribution of BANA scores

Site	Negative	Weak-positive	Strong-positive
Shallow Pocket	3[a]	29	20
Deep Pocket	2	20	30
Tongue	27	22	3
Saliva	33	15	4

[a] Number of samples.

differences between males and females, the unpaired *t* test was used.

Results

Table 1 presents the distribution of BANA scores from the four loci examined. Shallow and deep pockets yielded high scores, with 30 and 20 strongly positive results, respectively, as opposed to only 3 and 2 negative scores, respectively. In contrast, 27 and 33 samples taken from tongue dorsa and from saliva, respectively, were negative. In all instances, significant correlations ($p \leq 0.005$; Spearman) were found when BANA scores were compared among subjects. The highest association was between BANA tongue *vs.* saliva scores ($r = 0.589$; $p < 0.001$). Shallow pocket levels appeared to be more highly related to tongue scores ($r = 0.548$; $p < 0.001$), whereas deep pocket scores were more highly associated with saliva results ($r = 0.561$; $p < 0.001$). In order to obtain a wider overall distribution, the average score of the four BANA tests was calculated for each subject (Fig.).

Table 2 presents the mean ± standard deviation for the dental parameters and malodor measurements. Transformation of peak and steady-state VSC measurements to natural logarithms was carried out in order to obtain near-normal distributions. Among the three

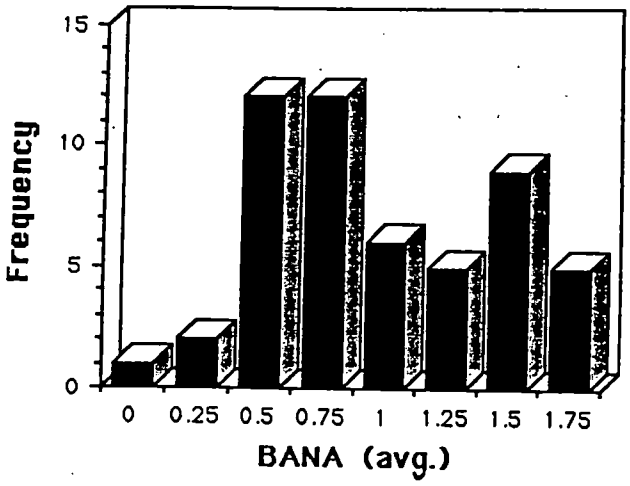

Figure. Frequency distribution of mean BANA scores.

organoleptic measurements, whole mouth odor scores were generally higher than saliva and tongue odor assessments (Table 2). Among the various measurements, the only significant difference between males ($N = 17$) and females ($N = 35$) was in mean BANA saliva scores (0.772 ± 0.187 for males, as opposed to 0.530 ± 0.090 for females, $p = 0.037$). No significant decrease was observed in mean peak of steady-state VSC measurements over the three days of the study ($p > 0.05$, ANOVA). Similarly, the time of day of measurement was not significantly related to any of the parameters.

The comparison between BANA scores and both oral malodor and dental parameters is summarized in Table 3. Associations with peak and steady-state VSC measurements were, for the most part, not significant. Only in the comparison of average BANA scores with steady-state

Table 2. Dental parameters and malodor measurements

Measurement	Mean ± Standard Deviation	No. of Observations
Plaque Index (PI)	1.2 ± 0.5	52
Gingival Index (GI)	1.1 ± 0.4	52
Mean Probing Depth	2.9 ± 0.7 mm	30
BANA (shallow pocket)	1.3 ± 0.6	52
BANA (deep pocket)	1.5 ± 0.6	52
BANA (tongue dorsum)	0.5 ± 0.6	52
BANA (saliva)	0.4 ± 0.6	52
BANA (average)	1.0 ± 0.5	52
Peak VSC	155 ± 62 ppb	51
Steady-state VSC	103 ± 38 ppb	51
Log Peak VSC	2.2 ± 0.14	51
Log Steady-state VSC	2.0 ± 0.14	51
Whole mouth odor (odor judge)	4.3 ± 2.0	52
Tongue odor (odor judge)	2.5 ± 1.3	52
Saliva odor (odor judge)	3.4 ± 2.5	52

Table 3. Spearman Correlation Coefficients comparing BANA results with oral malodor and dental parameters

	BANA (Shallow Pocket)	BANA (Deep Pocket)	BANA (Tongue)	BANA (Saliva)	BANA (Avg.)
Log Peak VSC	r = -0.019	0.151	0.134	0.059	0.098
	p = 0.894	*0.290*	*0.349*	*0.679*	*0.493*
Log Steady-state VSC	0.209	0.263	0.255	0.245	0.304
	0.142	*0.063*	*0.071*	*0.083*	*0.030*
Whole mouth	0.332	0.259	0.364	0.359	0.399
(odor judge)	*0.016*	*0.034*	*0.008*	*0.009*	*0.003*
Tongue	0.260	0.331	0.271	0.372	0.373
(odor judge)	*0.063*	*0.017*	*0.052*	*0.007*	*0.006*
Saliva	0.399	0.312	0.338	0.500	0.455
(odor judge)	*0.003*	*0.025*	*0.014*	*<0.001*	*0.001*
PI	0.325	0.101	0.293	0.329	0.306
	0.019	*0.476*	*0.035*	*0.017*	*0.028*
GI	0.452	0.282	0.481	0.406	0.472
	0.001	*0.043*	*<0.001*	*0.003*	*<0.001*
Mean Probing Depth	0.491	0.284	0.326	0.551	0.514
	0.006	*0.129*	*0.079*	*0.002*	*0.004*

measurements was a significant association (r = 0.304, p = 0.030) obtained. In contrast, organoleptic ratings of whole mouth, tongue, and saliva malodor were generally associated with BANA results. The highest association was found for BANA saliva scores *vs.* saliva malodor (r = 0.500; p < 0.001). Average BANA scores were highly associated (p ≤ 0.006) with all three organoleptic ratings. When BANA scores were compared with GI, highly significant correlations (p ≤ 0.001) were found for BANA average, BANA shallow pocket, and BANA tongue. Probing depth was highly associated with BANA shallow pocket (p = 0.006), BANA average (p = 0.004), and BANA saliva (p = 0.002) scores. Deep pocket BANA scores were not significantly associated with PI or probing depth.

Table 4 presents Pearson correlation coefficients comparing the oral-malodor-associated results as well as dental parameters. Among the dental parameters, GI scores provided the highest correlations with all odor-judge measurements (p ≤ 0.001). PI values were highly associated with GI, probing depth, and odor-judge saliva scores (p ≤ 0.001), but not with sulphide measurements. Correlations between GI and peak sulphide levels were higher than between GI and steady-state sulphide values (p = 0.017 and p = 0.053, respectively). Probing depth scores were more highly associated with steady-state than with peak sulphide scores (p = 0.008 and p = 0.050, respectively). All odor-judge parameters were highly associated with one another (p < 0.001). Correlations between both peak and steady-state sulphide scores *vs.* odor-judge ratings of whole mouth and saliva were highly associated (p < 0.001). Correlations between peak and steady-state sulphide scores and odor-judge tongue malodor scores were slightly lower (p = 0.003 and p = 0.013, respectively).

In an attempt to relate each of the three odor-judge measurements to both VSC levels and BANA (avg.) scores, step-wise forward multiple-regression analysis was carried out (Table 5). In all three cases (whole mouth, tongue, and saliva), both log peak monitor levels and BANA (avg.) scores factored into the equations, yielding, in all cases, highly significant correlations (multiple r = 0.568, 0.501, and 0.586, respectively, with significance levels of p = 0.0001, p = 0.001, and p < 0.0001, respectively). Following entry of log peak monitor levels into the regression equation, steady-state levels did not contribute significantly and were not entered.

Discussion

The Perioscan® BANA assay is a user-friendly chairside technique which has previously been shown to correlate with the levels of three specific oral microbial species whose presence reflects periodontal disease activity (Loesche, 1986; Loesche *et al.*, 1990a,b; Schmidt *et al.*, 1988). The results presented here provide further support for previously demonstrated associations between BANA test results and periodontal parameters (*e.g.*, GI, probing depth). Among the sites tested here, deep pocket BANA samples generally yielded less significant associations with dental parameters than BANA samples obtained from the other three sites. This may be associated with the low number (n = 2) of deep pocket samples which gave negative results, in agreement with previous data suggesting relatively low specificity (Loesche *et al.*, 1990b) of the BANA assay when plaque samples were compared with dental parameters. Results suggest that the BANA status of a single deep pocket may not be representative of parameters involving overall status.

The present study provides initial data comparing BANA scores with oral malodor parameters. Of considerable

Table 4. Pearson Correlation Coefficients comparing oral malodor-associated results and dental parameters

	PI	GI	Mean Probing Depth	Whole Mouth (odor judge)	Tongue (odor judge)	Saliva (odor judge)	Log Peak VSC	Log Steady-state VSC
PI	r = 1.00							
	p = 0.000							
GI	0.813	1.00						
	< 0.001							
Mean probing depth	0.577	0.668	1.00					
	< 0.001	*< 0.001*						
Whole mouth (odor judge)	0.383	0.536	0.581	1.00				
	0.003	*< 0.001*	*< 0.001*					
Tongue (odor judge)	0.383	0.468	0.466	0.725	1.00			
	0.003	*< 0.001*	*0.005*	*< 0.001*				
Saliva (odor judge)	0.433	0.583	0.513	0.730	0.599	1.00		
	0.001	*< 0.001*	*0.002*	*< 0.001*	*< 0.001*			
Log peak VSC	0.188	0.298	0.305	0.472	0.381	0.439	1.00	
	0.093	*0.017*	*0.050*	*< 0.001*	*0.003*	*0.001*		
Log steady-state VSC	0.092	0.230	0.436	0.404	0.313	0.413	0.718	1.00
	0.261	*0.053*	*0.008*	*0.002*	*0.013*	*0.001*	*< 0.001*	

interest is the finding that BANA results, although significantly associated in most cases with odor-judge ratings, were generally poorly associated with VSC levels. When multiple-regression analysis was performed with odor-judge measurements as the dependent variable, both peak VSC levels and BANA scores factored into the regression, yielding highly significant associations. These findings suggest that BANA scores are associated with some other factor(s), which helps account for odor-judge assessment of oral malodor, exclusive of VSC data. Possibly, micro-organisms associated with BANA assay contribute additional non-sulphide odorants, such as cadaverine (Goldberg *et al., 1994*), to the oral halitus.

In the present study, highly significant correlations were found for odor-judge scores as compared with dental parameters. Correlations between dental parameters and malodor levels have been shown in most previous studies (Rizzo, 1967; Tonzetich, 1973, 1977; Tonzetich and McBride, 1981; Coil and Tonzetich, 1985; Rosenberg *et al.*, 1991a; Mardinger *et al.*, 1992; Yaegaki and Sanada, 1992). The most striking associations obtained here were: (i) whole mouth odor assessment, as compared with mean probing depth ($r = 0.581$; $p < 0.001$); and (ii) saliva odor assessment, as compared with GI ($r = 0.583$; $p < 0.001$). It should be emphasized that, in the present study, saliva samples were collected following probing (even in those cases in which average probing depth was not recorded, probing was performed to assess deep *vs.* shallow pockets). The introduction of subgingival components into the saliva samples may have contributed to the highly significant correlations found with parameters involving saliva.

Quantification of oral malodor is a problem which has delayed scientific investigations into its causes and treatments. Oral malodor measurement by multiple judges is cumbersome and introduces problems of subjectivity and reproducibility. Agreement among judges, while highly significant, is usually

Table 5. Stepwise multiple-regression analysis of odor-judge assessments in terms of VSC and BANA (Avg.) scores

Dependent Variable	Intercept	Coefficient for Log Peak VSC	Coefficient for Log Steady-state VSC	Coefficient for BANA (avg.)	Multiple r
Whole mouth	-6.845	5.820	N.E.[a]	0.333	.0.568
	0.016[b]	*0.001*	*0.684*	*0.010*	*0.0001*
Tongue	-3.24	2.90	N.E.	0.217	0.501
	0.082	*0.010*	*0.460*	*0.012*	*0.001*
Saliva	-9.648	6.533	N.S.	0.507	0.585
	0.006	*0.002*	*0.796*	*0.002*	*<0.0001*

[a] Not entered into the equation due to lack of significance.

[b] Numbers in italics represent levels of significance.

moderate (Rosenberg *et al.*, 1991a,b; Rosenberg and McCulloch, 1992). One major difficulty is that once a subject has expelled breath for estimation by one judge, the halitus emitted subsequently for other judge(s) may differ in intensity and composition (Rosenberg and McCulloch, 1992). Intra-oral measurements with the sulphide monitor provide rapid quantitative data which are reproducible and sensitive, easy-to-use, and relatively inexpensive. Peak and steady-state sulphide monitor levels have been previously shown to provide highly significant correlations with odor-judge measurements (Rosenberg *et al.*, 1991a,b, 1992) but cannot be inferred to be a "gold standard" for bad breath measurement and thus to replace organoleptic assessment (Rosenberg and McCulloch, 1992). We have previously proposed that clinical measurement of oral malodor be conducted by organoleptic examination of oral malodor deriving from several loci, in conjunction with sulphide monitor measurements, as well as dental assessment (Rosenberg *et al.*, 1991a). The results of the present study suggest that the BANA test may be a simple, adjunct assay together with volatile sulphide determination in order to provide additional quantitative data which contribute to the overall association with odor-judge estimation. These techniques may prove advantageous in facilitating comparison of oral malodor investigations conducted at different locations by various investigators.

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ריח פה - הסיבות, הטיפול, הטעויות

ריח פה הוא בעיה נפוצה בין בוגרים בעולם המערבי, ואולי בכל העולם. אין ברשותנו הערכה מדויקת, אך קרוב לוודאי שכמעט לכל האנשים יש ריח פה בשעה כלשהי במשך היום.
למרות הסברה הרווחת בציבור, ריח פה כמעט אף פעם אינו תוצאה מבעיות עיכול. ברוב המקרים נעוץ מקור הבעיה בחלל הפה עצמו.
הגורם הוא תרכובות נדיפות המכילות גופרית, המיוצרות על-ידי חיידקים אגב פירוק שיירי אוכל ורוק, ואלה חבויים בגומחות שונות בחלל הפה, כגון כיסי החניכיים, גב הלשון ובין השיניים. תנאים נוספים הם התייבשות בפה (למשל ירידה בזרימת רוק בזמן השינה), וכן תהליכי דלקת פעילים ברקמות החניכיים.
אם מקור הריח הוא אכן בפה מומלץ לצחצח שיניים היטב, להבריש את הלשון, להשתמש בחוט דנטלי ולהריחו לאחר מעבר בין כל זוג שיניים. מי פה יעילים יכולים לעזור בהפחתת הריח, אך אין הם תחליף לצחצוח וניקוי מכני.
במקרים אחרים מקור הריח במערות האף, בשל בעיות רפואיות דוגמת סינוסיטיס ופוליפים. הבעיה אינה מלווה לעתים קרובות בממצאים פתולוגיים. הריח יכול לנבוע למשל מהתייבשות הריריות באף, ובמקרה כזה קורה שהרופא אינו מוצא דבר. במקרים נדירים מוצאים בתוך האף עצמים הגורמים לריח, בייחוד אצל ילדים אשר עלולים לדחוף כל דבר לאף. לפני שנה נמצא באפה של בחורה בת 28 חרוז שנתקע והסתייד שם במשך כ-25 שנים, והוא היה הגורם לריח. במקרה זה אותרו שלושה סוגי ריח: ריח שמקורו בפה, ריח עשן סיגריות וריח שמקורו באף, וזה האחרון הוביל למציאת האוצר.
הריח הכללי נוצר עקב תרומות של משטחים ואזורים שונים בחלל הפה והאף. בבדיקה יודעים לפעמים מייד מה מקור הבעיה, ולעתים יש צורך לדגום ממשטחים שונים ולגלות את מקור הריח. ריח שמקורו רק בפה מסמן קיום בעיה בפה או בגרון. ריח שמקורו רק באף מסמן בעיות במערות האף. ריח שמקורו בשניהם גם יחד יכול לסמן תערובת בעיות או בעיה יחידה בחלקים פנימיים יותר של הגוף - סימפונות, ריאות, כבד, כליות, בעיות מטבוליות, ובמקרים נדירים אפילו סוגים מסוימים של סרטן.

אצל כשליש מהפונים לא נמצאים ממצאים אובייקטיביים של ריח, למרות תלונתם. קשה לשכנע אדם המאמין במשך עשרים או שלושים שנה שהוא סובל מריח פה חריף, שבעצם אין לו בעיה כזו.

כאשר מקור הבעיה הוא בחלל הפה ניתן ייעוץ ספציפי לגבי היגיינה או טיפול חניכיים. ריח פה מהחניכיים יכול להזהיר מפני מחלה מתפתחת, כאשר המצב עדיין הפיך, ומאפשר טיפול. כאשר מקור הריח באף יש לפנות לרופא מומחה. לעתים מקור הריח אינו ברור, ואז יש לערוך סדרת בדיקות לאיתור הבעיה.
קיים גם מצב שכיח, בו מדובר בבעיה פסיכולוגית. אצל כשליש מהפונים לא נמצאים ממצאים אובייקטיביים של ריח, למרות תלונתם. הטיפול בבעיות פסיכולוגיות כאלה יכול להיות מסובך בהרבה מאשר הפתרונות הרפואיים שצויינו. קשה לשכנע אדם המאמין במשך עשרים או שלושים שנה שהוא סובל מריח פה חריף, שבעצם אין לו בעיה כזו. הנסיבות הפסיכולוגיות הגורמות לריח פה מדומה כזה אינן נהירות עדיין.
רבים פונים לייעוץ משום שריח פה אינו ניתן למדידה והערכה על-ידי הפרט עצמו. הפונים רוצים לקבל משוב אובייקטיבי, ולעתים מגיעים בעקבות הערות של בני משפחה. העיסוק בנושא בתרבות המערבית זנות. הרפואה המודרנית אינה עוסקת בו ואינה מצליחה להציע פתרונות, בעוד שברפואה המזרחית ריח הוא נושא מרכזי באבחון. כל מה שדרוש, בעצם, הוא לעשות שימוש בחוש הריח, הפועל אוטומטית - שהרי כל אדם מריח כאשר הוא נושם.

בתמונה: מכשיר שפותח למדידה כמותית של ריח פה

פרופ' רוזנברג-נבו הוא מיקרוביולוג ומנהל מרכז ייעוץ לבעיות ריח פה במרכז רפואי בזל.

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Kinetic analysis of ammonium sulfate dependent aggregation of bacteria

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COLLOIDS AND SURFACES B

Kinetic analysis of ammonium sulfate dependent aggregation of bacteria

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Abstract

Solutions of ammonium sulfate (AMS) have been found to aggregate bacteria in a quantifiable and reproducible manner. The rates of aggregation promoted by the AMS of several bacteria obeyed pseudo-first-order kinetics. There was a high correlation between the rate of AMS aggregation of bacteria and the rate of adhesion to hexadecane by the bacteria. Rates of aggregation depended on AMS concentration, cell age, bacterial growth conditions and density of the cell suspension. Plots of rate constants of aggregation by AMS against AMS concentrations gave "S" shaped curves, implying cooperative effects. Addition of mannose did not alter the rate of AMS dependent aggregation of a Fim A strain of *Escherichia coli*, suggesting that ligands on the surface proteins do not interfere with the process of aggregation. Previous studies have implied that the slide aggregation of bacteria by AMS solutions reflects bacterial hydrophobocity. It is now proposed that rate constant determinations, based on loss of opacity of cellular suspensions in AMS, constitute a convenient means of studying bacterial hydrophobicity.

Keywords: Ammonium sulfate; Hexadecane; Hydrophobicity; Rate assay

1. Introduction

Several years ago, Lindahl et al. [1] observed that some bacteria were more readily aggregated by ammonium sulfate (AMS) solutions than others. Based on several criteria, the ease of "salting-out" of bacteria is a function of the hydrophobicity of the cells [2]. The assay of Lindahl et al. employed a glass slide on which cells and AMS were mixed. No effort was made to quantify the AMS-dependent aggregation. Later, Rozgonyi et al. [3] were able to increase the sensitivity of detection by adding a chromophoric dye to the AMS–bacteria mixtures. The aggregation of bacteria by a variety of agents can be measured by determining the loss of turbidity of cellular suspensions. For example, the addition of α-1,6-glucans to suspensions of *Streptococcus cricetus* or *S. sobrinus* results in cellular aggregation which can be monitored by spectrophotometric measurements. Plots of the log of the change in turbidity vs. time yield pseudo-first-order rate constants, thereby affording a means of quantifying the aggregation [4]. In the present studies, it was determined that the aggregation of bacteria by AMS could be quantified by rate measurements. The rates of aggregation were dependent on AMS concentration, bacterial density, age of culture and other variables. It was also found that the rates of aggregation of bacteria by

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AMS (rate-SAT) were proportional to the rates of adhesion of the bacteria to hexadecane, a hydrophobic solvent frequently employed in assaying microbial cell surface hydrophobicity [5–13].

2. Materials and methods

2.1. Bacterial strains

Streptococcus sanguis 10556, *Streptococcus gordonii*, *Streptococcus oralis*, *Streptococcus mutans* 10449, *Streptococcus mutans Ingbritt* 162, *Streptococcus cricetus* HS6, *Streptococcus cricetus* AHT, *Streptococcus cricetus* E49, *Streptococcus sobrinus* DB13, *Streptococcus sobrinus* 33478, *Streptococcus sobrinus* 6715, *Acinetobacter calcoaceticus* MR-481, *Acinetobacter calcoaceticus* RAG-1, *Bacillus subtilis* 168, *Pseudomonas aeruginosa* 1012, *Pseudomonas aeruginosa* 44, and *Escherichia coli* were used in this study. For daily use, bacteria were stored as turbid suspensions in Trypticase soy broth (TSB) or brain heart infusion broth (BHI) at −20°C. The sterile medium was inoculated with 0.1 ml aliquots of the refrigerated stock.

2.2. Media preparation

BHI, nutrient broth (NB), TSB (all from Difco Laboratories, Detroit Michigan) and Penassay broth (from BBL, Cockeysville, MD) were used as growth media. In order to eliminate potential sucrose or α-1,6-glucan contaminants in TSB, the medium was treated with dextranase (1.6 mg/g of dry medium, Sigma, St. Louis, MO) at 37°C for 2 h and invertase (0.2 mg/g of dry medium, Sigma) at 55°C for 2 h prior to autoclaving. All the media were autoclaved before inoculation of bacteria.

2.3. Growth conditions

The cultures were incubated for 18 h at 37°C or room temperature and the cells were then harvested (Table 1 outlines the growth conditions for all the bacteria). The cells were suspended in PBS buffer (20 mM potassium phosphate, 0.15 mM NaCl, pH 7.2), centrifuged (6000 *g*), and washed twice with the same buffer. Bacterial cell pellets were suspended in PBS at appropriate cell densities, before salt aggregation tests (SAT) and microbial adhesion to hydrocarbon (MATH) [5] tests were performed.

For *B. subtilis* 168, vegetative cells and spores were prepared. To produce the spores, 20 ml Penassay broth was inoculated with bacteria from an AK agar and incubated aerobically with vigorous shaking at 37°C for 16–18 h. Three milliliters of this culture was transferred to 200 ml BHI medium, 0.5% (wt/vol) yeast extract, 1.0 mM Ca^{2+}, and 50 μM Mn^{2+} in a 2800 ml flask [13]. The cultures were then shaken vigorously at 25–27°C for 48 h to induce sporulation. At appropriate intervals, samples were removed, centrifuged at 6000 *g* for 10 min and washed twice in PBS. When sporulation was detected under the microscope, 20 ml portions of the culture were transferred to a 250 ml flask and added to 200 ml PBS and lysozyme (50 μg ml^{-1} final concentration). The mixture was incubated at 37°C for 1 h. A Gram stain was made to examine the release of spores from the sporangia with the microscope. The spores were then extracted by centrifugation, and washed twice and suspended in PBS. The absorbance was adjusted to 0.8, 1, 1.5, 1.8 at 540 nm in a spectrophotometer. Cells were obtained by aerobic growth in Penassay broth.

2.4. Salt aggregation test (SAT)

Two milliliter portions of the bacterial suspensions were adjusted to optical densities of 0.8, 1, 1.5 and 1.8 at 540 nm in PBS, and added to an equal volume of ammonium sulfate to produce final concentrations of 0.5, 1, 1.5 and 2 M, respectively. Control tubes contained 2 ml PBS. The mixtures were rapidly vortexed (15 s) and the decrease in optical density was continually monitored by the spectrophotometer. Rate constants were obtained from the slopes of first-order plots of ln A/A_o (A = observed optical density, A_o = optical density at time zero) vs. time in min. The coefficient of each experiment was recorded and computed as K.

2.5. MATH (microbial adhesion to hydrocarbons)

MATH is the general method for measuring bacterial adhesion to liquid hydrocarbons [5].

Table 1
Bacterial growth conditions

Bacterium	Medium[a]	Temperature (°C)	CO_2	Shaking
Bacillus subtilis 168	BHI	37	–	+
Escherichia coli	BHI	37	–	+
Pseudomonas aeruginosa 44	TSB	37	+	+
Pseudomonas aeruginosa 1012	TSB	37	+	+
Acinetobacter calcoaceticus RAG-1	BHI	37	–	+
	BHI	37	–	–
	BHI	Room temperature	–	+
	TSB	37	–	+
	Penassay broth	37	–	+
	NB	37	–	+
Acinetobacter calcoaceticus MR-481	BHI	37	–	+
Streptococcus cricetus AHT	TSB	37	+	–
Streptococcus cricetus HS6	TSB	37	+	–
Streptococcus cricetus E49	TSB	37	+	–
Streptococcus gordonii	TSB	37	+	–
Streptococcus mutans 10449	TSB	37	+	–
Streptococcus mutans Ingbritt 162	TSB	37	+	–
Streptococcus oralis	TSB	37	+	–
Streptococcus sanguis 10556	TSB	37	+	–
Streptococcus sobrinus 6715	TSB	37	+	–
Streptococcus sobrinus DB13	TSB	37	+	–
Streptococcus sobrinus 33478	TSB	37	+	–

[a] NB, nutrient broth; BHI, brain heart infusion broth; TSB, Trypticase soy broth.

n-Hexadecane was used to determine the surface hydrophobicity of the cells. In this test, the 3.0 ml cells (adjusted to optical densities of 0.8, 1.0, 1.5, and 1.8 at 540 nm in a spectrophotometer) were mixed with 0.6 ml hexadecane by vigorous vortexing. The control tubes contained a cell suspension in 3 ml PBS only. Adhesion was expressed as the percentage of decrease in turbidity following the mixing procedure compared to that of the corresponding cell suspension prior to the assay [5], or by calculating the rates of decrease in opacity [6,9].

3. Results

3.1. Turbidimetric changes in bacteria following addition of ammonium sulfate

Cultures of bacteria were obtained in the late exponential growth phase. The cells were centrifuged, washed twice with PBS and suspended in the buffer at an optical density of 0.8–3.0 at 540 nm. Various concentrations of AMS were added to the suspensions, which were then vortexed rapidly and their turbidities observed as a function of time. Fig. 1 shows the aggregation of *A. calcoaceticus* strains RAG-1 and MR-481 by AMS. Strain RAG-1 was readily aggregated by an AMS concentration of 1.0 M, whereas strain MR-481 was not. When both strains were vortexed with hexadecane in the standard MATH test, it was found that RAG-1 adhered to the hydrocarbon (Fig. 2). Results from the laboratory of Rosenberg and Rosenberg [14] have shown that strain RAG-1 is highly hydrophobic, whereas strain MR-481, a mutant derived from RAG-1, is non-hydrophobic. Settled suspensions were observed by light microscopy and found to contain aggregates, confirming that the salt aggregated the bacteria.

When another bacterium, *Escherichia coli*, was mixed with AMS solutions it also aggregated, even

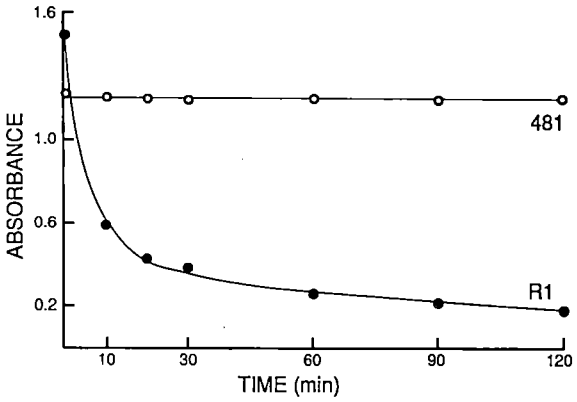

Fig. 1. Aggregation assay of *Acinetobacter calcoaceticus* strains RAG-1 and MR-481 by the salt aggregation test. Cells were from shaken cultures in BHI broth at 37°C (Table 1). Prior to mixing with 2.0 M AMS the cells were centrifuged, washed twice in PBS and suspended at a density of 3.0 absorbance units at 540 nm.

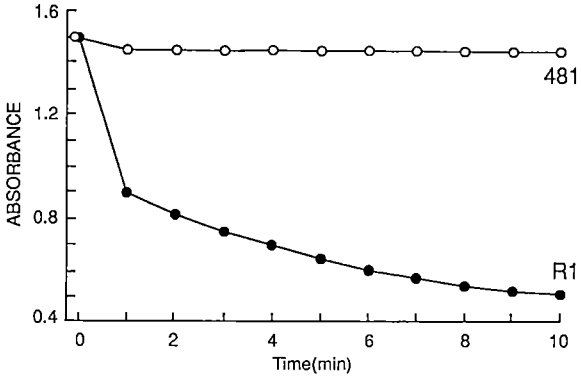

Fig. 2. Adhesion of *Acinetobacter calcoaceticus* strains RAG-1 and MR-481 to hexadecane. Cells were obtained as described in Fig. 1 and adjusted to an absorbance of 1.5 at 540 nm in PBS. After addition of hexadecane (0.2 ml hexadecane per ml cell suspension), the mixtures were vortexed for 1.0 min, and absorbance readings of the aqueous suspensions were then taken. Intermittent vortexing was continued for up to 10 min.

at 0.5 M AMS. The rate of aggregation of *E. coli* was significantly higher at 2 M, compared to 1.5 and 1.0 M AMS (Fig. 3). This strain of *E. coli* possessed the Fim A adhesin, which is a protein located at the very tips of fimbriae enabling the bacteria to bind mannose-containing glycoconjugates. When the SAT was performed in the presence of up to 200 mM mannose, there were no changes in the aggregation rates of the bacterium induced

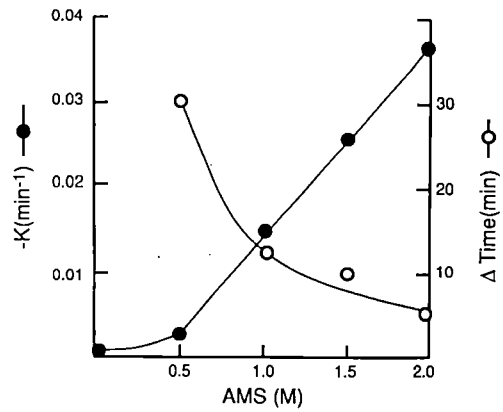

Fig. 3. Aggregation of *Escherichia coli* by AMS solutions. *E. coli*, from a late exponential phase culture (Table 1), was centrifuged and washed twice in PBS. The cells were adjusted to an absorbance of 2.0 before mixing with equal volumes of AMS. ΔTime refers to the time required for an exponential decrease in absorbance to occur after the addition of AMS.

by AMS. For *E. coli*, delays in aggregation occurred following the addition of AMS (Fig. 3). The greater the AMS concentration, the shorter the lag time required for observing turbidity changes. In fact, most of the bacteria used in this study, except *A. calcoaceticus* RAG-1, exhibited lag times in aggregation on addition of AMS solutions.

3.2. Measurement of rates of aggregation induced by AMS

It is often convenient to plot absorbance against time in terms of pseudo-first-order rate plots. We attempted to find out whether, at a given AMS concentration, one can distinguish between various species or strains in terms of their aggregation rate constants. Fig. 4 shows first-order plots for the aggregation of several bacteria compared with the rates of removal of the bacteria from a PBS suspension by hexadecane. It is clear that there is a strong quantitative relationship between the rates of AMS-promoted aggregation and adhesion to hexadecane. Some bacteria, such as *B. subtilis*, are clearly hydrophilic, as they have no tendency to be aggregated by AMS or to bind to the hydrocarbons. Other bacteria, in contrast, are highly hydrophobic and are readily aggregated by AMS and adhere to hexadecane.

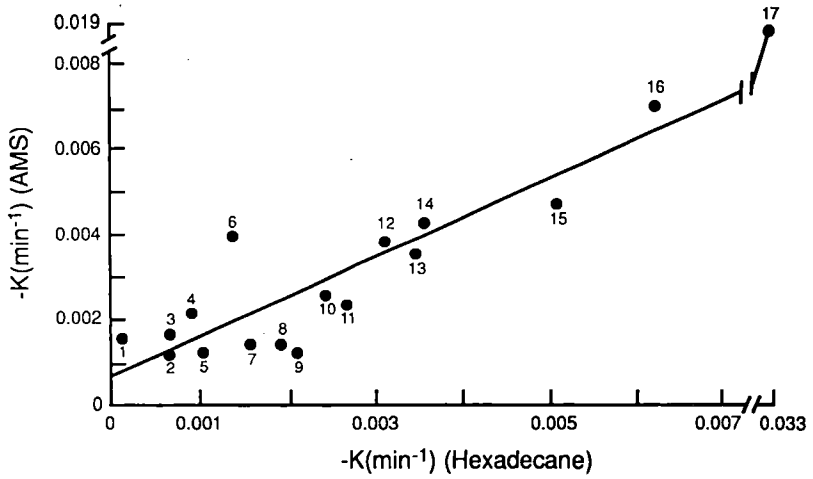

Fig. 4. Rates of cellular aggregation induced by AMS plotted against rates of adhesion to hexadecane. Cells were from late exponential cultures (Table 1) at 37°C. The final concentration of AMS was 1.0 M, whereas the ratio of cells to hexadecane was 1.0:0.2. All cell suspensions were adjusted to 1.5 absorbance units prior to addition of an equal volume of AMS or of hexadecane. The data were subjected to a linear regression, yielding a correlation coefficient of 0.83 ($p<0.01$). 1, *B. subtilis* 168; 2, *P. aeruginosa* 44; 3, *P. aeruginosa* 1012; 4, *A. calcoaceticus* MR-481; 5, *S. sanguis*, 10556; 6, *S. gordonii*; 7, *S. oralis*; 8, *S. mutans* 10449; 9, *S. mutans* Ingbritt 162; 10, *S. sobrinus* 33478; 11, *S. sobrinus* DB13; 12, *S. sobrinus* 6715; 13, *B. subtilis* spores; 14, *S. cricetus* E49; 15, *S. cricetus* HS6; 16, *S. cricetus* AHT; 17, *A. calcoaceticus* RAG-1.

When a single batch of cells was employed, the reproducibility of aggregation rates did not vary by more than ±5%. As discussed below, cell age and culture conditions influence the AMS-dependent aggregation rates of bacteria.

3.3. Influence of cell age on aggregation by AMS of Pseudomonas aeruginosa

Wentworth et al. [15] speculated that as *P. aeruginosa* ages, some of the cells lyse, releasing cytoplasmic lectins that may act as adhesins. These adhesins were thought to initially bind to the cell surfaces of any intact *P. aeruginosa*, thereby promoting the adhesion of the bacteria. It was desirable, therefore, to assay exponential and aged cultures of *P. aeruginosa* with the SAT in order to detect any surface changes in the organism. Fig. 5 shows that as *P. aeruginosa* 1012 progressively ages, the cells become more amenable to aggregation by AMS. It is impossible to describe the nature of the new hydrophobins, or to determine if they are lectins. Nevertheless, when studying AMS–dependent bacterial aggregation, it is necessary to carefully standardize the culture conditions.

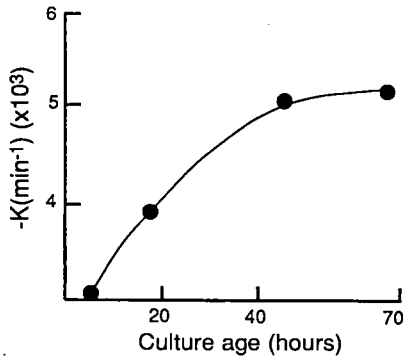

Fig. 5. Aggregation of *Pseudomonas aeruginosa* 1012 by AMS as a function of cell age. Prior to mixing with an equal volume of 3.0 M AMS, *P. aeruginosa* cells were grown with shaking at 37°C (Table 1) and washed twice in PBS. Pseudo-first-order rate constants were obtained from cultures of various ages as described.

3.4. Culture conditions and SAT

A. calcoaceticus RAG-1 is known to be highly hydrophobic as well as possessing surface fimbriae [14]. Fimbriae are usually hydrophobic, so fimbriae-rich bacteria are generally regarded as adhering more readily to hexadecane [7]. In

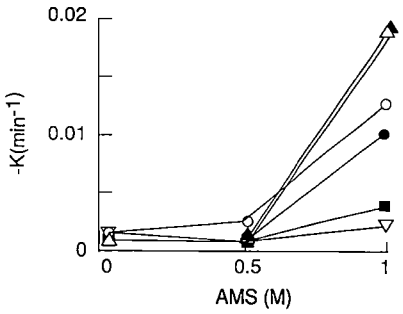

Fig. 6. Influence of growth conditions on the tendency of *Acinetobacter calcoaceticus* RAG-1 to be aggregated by AMS. Cells were removed from their respective growth media by centrifugation, washed twice in PBS and finally suspended at an absorbance of 1.6 prior to mixing with an equal volume of 2 M AMS. *K*-values were the first-order rate constants determined by absorbance vs. time plots. BHI broth at 37°C with shaking (■); BHI broth at 37°C without shaking (△); BHI broth at 22°C without shaking (▲); NB at 37°C with shaking (●); TSB at 37°C with shaking (○); Penassay broth at 37°C with shaking (▽).

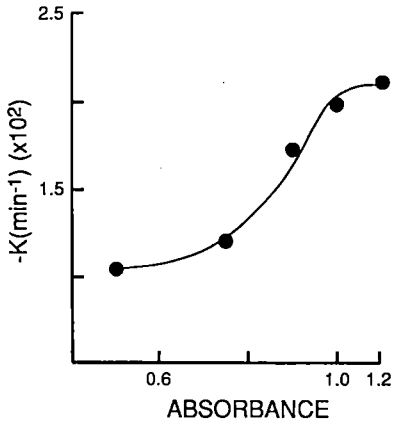

Fig. 7. Influence of cell density on the aggregation of a bacterial suspension by AMS. Cell suspensions of *A. calcoaceticus* RAG-1 were adjusted to various optical densities and mixed with an equal volume of 2 M AMS. Rate-SAT (*K*-values) were determined from the first-order plots.

addition, shaken cultures of *Enterobacteriaceae* tend to be less fimbriated than static cultures. Fig. 6 shows the influence of growth conditions on the SAT of *A. calcoaceticus* RAG-1. The two static cultures (cells grown at room temperature and at 37°C without shaking) had the highest tendency to be aggregated by 1.0 M AMS. The same strain grown with shaking had a high degree of AMS-dependent aggregation, as did cells grown with shaking in NB, Penassay broth and TSB. However, the cells from the NB and Penassay broth cultures exhibited a delay in aggregation. These results reveal that culture conditions, such as type of nutrient and degree of aeration, can influence the activities of *A. calcoaceticus* R1 in AMS solutions.

3.5. Cell density and SAT

A. calcoaceticus RAG-1 was grown in BHI broth at 37°C with shaking (Table 1) to give the late exponential phase. The cells were harvested, washed twice in PBS and then suspended at various cell densities, as determined by spectrophotometric readings of diluted samples. Equal volumes were mixed with 2 M AMS and vortexed, and SAT-rates were determined. When K (min^{-1}) was plotted against cell density, it was seen that at the lower cell densities SAT-rates were low, but increased disproportionally at higher cell densities (Fig. 7). Another bacterium, *S. sobrinus* 6715, also yielded an S-shaped curve under similar conditions, which implies cooperative effects. The results show that for the purposes of comparison between cell types, growth conditions, etc., the cell densities (or the number of cells) must be carefully standardized.

4. Discussion

The results of this research can be summarized as follows: (i) ammonium sulfate aggregates bacteria in a reproducible and measureable manner; (ii) the rate of aggregation is dependent on AMS concentration, the age of the culture, the growth medium of the cells, and cell density; (iii) the rate of aggregation induced by AMS is proportional to the rate of binding of the cells to hexadecane; (iv) ligands attached to the surface protein on the cell do not appear to change the rate of AMS-induced cellular aggregation, at least for *E. coli*. Because of the close correlation between aggregation brought about by AMS solutions and the binding of the cells to hexadecane, it is suggested that SAT-rate assays may provide a new means of monitoring cell surface hydrophobicity.

In previous SAT assays, cells have been mixed with AMS solutions on microscope slides and observed for aggregation [1,16–18]. These SAT assays are rapid and reproducible, but suffer from the lack of a quantifiable variable. In the present research, actual rates of aggregation of various bacteria were determined by turbidity measurements and calculated first-order rate constants. When care is taken to standardize growth conditions, the rate constants may serve as a quantitative index of cellular hydrophobicity. It must be emphasized that in order to ascribe AMS-dependent aggregation to cellular hydrophobicity, one must accept that adhesion to hexadecane also reflects hydrophobicity. The studies of Rosenberg et al. [5], employing various methods to probe hydrophobicity, suggest MATH as a rapid and convenient assay of microbial hydrophobicity. Although several studies have shown a correlation between MATH and other methods for measuring bacterial hydrophobicity [2,8,10,11], the results have not always been conclusive. Recently, Geertsema-Doornbusch et al. [19] were able to observe only limited adhesion of various streptococci to hexadecane when the ionic strength was low. It is likely that at low ionic strengths, electrostatic effects between cells and hydrocarbon are more energetically favored than cell–hydrocarbon interactions. The MATH measurements undertaken in the present study employed a sufficiently high ionic strength to afford cell–hydrocarbon binding. Based on the close relationship between SAT and MATH aggregation rates (Fig. 4), it seems likely the former reflect cellular hydrophobicities.

The mechanism of SAT is not completely resolved, but is probably related to the well-known ability of the reagent to "salt-out" proteins [20,21]. Some proteins are salted out by low concentrations of AMS, whereas others require much higher concentrations. The AMS may abstract water from the microbial surface and neutralize electrostatic charges on the cells, thereby allowing the hydrophobic effect to dominate the cell–cell interaction [19]. Cells with a higher degree of hydrophobicity should be more readily aggregated by AMS than hydrophilic cells.

The observation that "threshold" concentrations of AMS are required to initiate aggregation (Figs. 3 and 6) suggests that cooperative interactions may be involved in the aggregation process. Cooperative adhesion, in which the adhesion of a bacterium to a substratum enhances the adhesion of other bacteria to the same substratum, has been reported for several bacteria [12,22]. Further, the rates of AMS-dependent aggregation of *S. sobrinus* were strongly dependent on cell density (Fig. 7). The characteristic "S"-shaped curve for AMS aggregation is suggestive of cooperative effects. There is no obvious explanation of the involvement of such cooperative effects in the AMS-mediated aggregation of bacteria. Others have previously noted that AMS only aggregates bacteria at relatively high cell densities [3,16–18].

The SAT, as described here, should be useful under a variety of conditions. The rate-SAT can be used to conveniently monitor the presence of hydrophilic mutants of hydrophobic parents. It may be possible to separate microbial mixtures, based on SAT, with aggregation occurring more easily for those with greater hydrophobic character. The rate-SAT is non-destructive, and once cells are aggregated they can be recovered by centrifugation and washed. Further, the suspensions may contain ligands such as mannose for the Fim A of *E. coli*. Although mannose did not modify the aggregation of *E. coli* by AMS, it seems possible that α-1,6-glucans may impose hydrophilic properties on hydrophobic oral streptococci. These studies are the first to use rate measurements for SAT. It was shown that rate-SAT is proportional to rate-MATH. We propose that rate measurements of SAT provide a new means of studying microbial cell surface hydrophobicity. No claims are made that rate-SAT measurements are superior to any other method for determining hydrophobicities, as each procedure has its own advantages.

Acknowledgments

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Self-estimation of Oral Malodor

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Abstract. Bad breath (halitosis, oral malodor) is a common condition, usually the result of microbial putrefaction within the oral cavity. Often, people suffering from bad breath remain unaware of it, whereas others remain convinced that they suffer from foul oral malodor, although there is no evidence for such. The purpose of the present investigation was to determine whether objective self-measurement of oral malodors is possible. Each of 52 volunteers was asked to sample the odor from his/her mouth, tongue, and saliva. Results were compared with (i) self-assessments prior to (preconception) and following (post-measurement) self-measurements; (ii) odor judge scores; (iii) dental measurements (plaque index, gingival index, and probing depth); (iv) volatile sulphide levels; (v) salivary cadaverine levels; and (vi) intra-oral trypsin-like activity. Among the self-measurements, only saliva self-scores yielded significant correlations with objective parameters. Despite the partial objectivity of saliva self-estimates, subsequent post-measurement self-assessments failed to correlate with objective parameters. The results suggest that (i) preconceived notions confound the ability to score one's own oral malodors in an objective fashion; and (ii) partial objectivity can be obtained in the case of saliva self-measurement, presumably because the stimulus is removed from the body proper.

Key words: halitosis, self-estimation, odor judge, dental indices, psychopathology.

Introduction

Although bad breath (halitosis, oral malodor, *fetor ex ore*) is among the most common of medical conditions, relatively little is known about this embarrassing and sometimes debilitating problem (Kleinberg and Westbay, 1990; Scully *et al.*, 1994). In most instances, bad breath stems from putrefactive microbial activity within the oral cavity itself and can be alleviated by proper oral hygiene and dental care (Spouge, 1964; Tonzetich, 1977; Attia and Marshall, 1982; Rosenberg *et al.*, 1991a; Yaegaki and Sanada, 1992). Bad breath intensity, as measured by judges, is significantly associated with intra-oral levels of volatile sulphides (Tonzetich and Richter, 1964; Schmidt *et al.*, 1978; Rosenberg *et al.*, 1991a,b; De Boever *et al.*, 1994).

One of the most intriguing problems regarding bad breath is the apparent inability of knowing whether one has it, and to what extent. Many people emit bad breath for years without being at all aware of it. Others greatly overestimate their own oral malodor and are consequently prone to obsessive behaviour, avoid social interactions, and may even contemplate suicide (Spouge, 1964; Davidson and Mukherjee, 1982; Hawkins, 1987; Iwu and Akpata, 1989; Iwakura *et al.*, 1994; Rosenberg and Leib, 1995).

It has previously been postulated that we do not notice our own oral malodor because we become adapted (habituated) to it (Engen, 1982; Spouge, 1994). However, this does not prevent someone with bad breath from smelling it in others (Rosenberg and McCulloch, 1992). Furthermore, subjects visiting oral malodor clinics sometimes claim to be able to smell their own malodor by a variety of techniques, *e.g.*, cupping hands over mouth and nose, licking hands, smelling the telephone mouthpiece, smelling the dental floss and/or saliva, rubbing fingers across the gums, and breathing beneath a blanket (Rosenberg and Leib, 1995). The main purpose of the present study was to determine whether self-measurements of this kind may help subjects obtain more objective insight into the extent of their own oral malodor. To this end, 52

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subjects were asked to measure and estimate their own whole mouth, tongue, and saliva malodors. Self-assessments prior to and following the various self-measurements were also recorded. Results were compared with (i) odor judge scores and volatile sulphide levels (Rosenberg *et al.*, 1991a,b); (ii) laboratory tests (Goldberg *et al.*, 1994; Kozlovsky *et al.*, 1994); (iii) dental indices (Rosenberg *et al.*, 1991a); and (iv) psychopathological profile (Derogatis *et al.*, 1973).

Materials and methods

Patient population

The data reported here constitute the main findings of a comprehensive study on parameters related to bad breath in 52 (35 females, 17 males; mean age, 37.9 years) subjects, 43 of whom complained of having bad breath. Other aspects of the study included the potential role of diamines in bad breath (Kozlovsky *et al.*, 1994) and the BANA test as a potential adjunct assay for oral malodor determination (Goldberg *et al.*, 1994). The human subjects protocol was appropriately approved by an internal review panel, and informed consent was obtained.

Self- and odor judge assessments

For self-assessments, subjects were asked to score their own oral malodor on a continuous 10-cm visual analogue scale (VAS) marked on each end as "no odor" and "extremely foul odor", respectively. Five self-assessments were made: (i) Preconception score—prior to measurement, subjects were asked to score the level of bad breath which they thought they had at that time; (ii) whole-mouth malodor score—subjects were instructed to smell the odor emanating from their entire mouth by cupping their hands over mouth and nose, exhaling through the mouth, and breathing in through the nose; (iii) tongue malodor score—subjects were asked to extend their tongue and lick their wrist in a perpendicular fashion (Odor was judged by smelling the wrist after 5 sec at a distance of *ca.* 3 cm.); (iv) saliva malodor score—saliva samples (*ca.* 1 mL), obtained by expectoration following the dental examination, were allowed to stand in closed Petri dishes for 5 min at 37°C and were then presented for odor assessment at a distance of *ca.* 4 cm; (v) post-measurement score—Immediately following the various self-assessments, subjects were again asked to rate their own oral malodor. In each instance, subjects were blinded to all previous scores. Finally, as a control to assess the subjects' scoring of a given foul odor, chicken-dung-based fertilizer (Kuftigal, Haifa, Israel) in aqueous suspension was presented to the subjects and the odor judge in an opaque sniff bottle and scored on the 10-cm VAS.

Organoleptic measurements of whole mouth, tongue, and saliva were made by an odor judge (MR) whose scores have previously been shown to correlate with those of other judges, as well as with volatile sulphide levels (Rosenberg *et al.*, 1991a,b). Odor judge assessments were carried out alongside the subjects' self-measurements, and scored analogously. For whole-mouth odor, the subject was asked to breathe out from the mouth, at *ca.* 10 cm from the nose of the judge; tongue and saliva odor were scored extra-orally by the odor judge in the same manner as that used for subject self-assessment, directly following subject's appraisal. The odor judge was blinded to self-assessments as well as to all other parameters, including his own previous scores.

Laboratory measurements

Physiological measurements included: (i) intra-oral measurement of peak and steady-state levels of volatile sulphides (Rosenberg *et al.*, 1991a,b) obtained by means of an Interscan (Chatsworth, CA, USA) model 1170 portable sulphide monitor fitted with disposable plastic straws at the input port, and connected to a pen recorder (Linear, model 285, Reno, Nevada); (ii) cadaverine levels measured in the expectorated saliva samples (Goldberg *et al.*, 1994); and (iii) trypsin-like activity, measured by the BANA test and averaged from four oral samples taken from deep pocket, shallow pocket, tongue dorsum, and saliva expectorate, respectively (Kozlovsky *et al.*, 1994).

Clinical measurements

Dental parameters consisted of plaque and gingival indices as well as probing depth. Participants were scored for plaque accumulation and gingival health of all tooth surfaces (including wisdom teeth) according to the Plaque Index (PI; Silness and Löe, 1964) and the Gingival Index (GI; Löe and Silness, 1963). Results presented represent the mean of all surfaces recorded. Mean probing depth measurements were recorded for all available sites (Kozlovsky *et al.*, 1994). Probing depth data were recorded for 30 of the subjects. Since odor levels of saliva are usually low, the saliva samples were obtained following the dental examination, for maximum odor level.

Psychopathological profiles

Psychopathological profiles were obtained by the SCL-90 (Self-Report Symptom Inventory Check List) questionnaire. The SCL-90 has been successfully applied in a variety of medical- and dental-related investigations (Derogatis *et al.*, 1973; Guy, 1976). The profile includes the dimensions of somatization, obsession-compulsion, interpersonal sensitivity, depression, anxiety, hostility, phobic anxiety, paranoid ideation, and psychoticism, as well as global scores designated as general symptomatic index, positive symptom total, and positive symptom distress index.

Statistical analyses

Volatile sulphide and salivary cadaverine levels were log-transformed so that near-normal distributions would be obtained. Parameters were compared by Pearson correlation analysis, paired *t* test, and stepwise forward multiple-regression analysis. Statistical analyses were performed with SPSS software (SPSS Inc., Chicago, IL).

Results

Table 1 compares odor assessments (both judge and self-estimates) from each of the three sources (whole mouth, tongue, and saliva) with laboratory tests and clinical parameters. Throughout, odor judge assessments of whole mouth, tongue, and saliva odor were significantly associated with laboratory measurements and dental indices (peak and steady-state volatile sulphide concentrations, salivary cadaverine levels, plaque index, gingival index, probing

Table 1. Pearson analysis of judge scores and self-assessments as compared with physiological and dental parameters

	Volatile Sulphides (peak level)[a]	Volatile Sulphides (steady-state level)[b]	Cadaverine[c]	Plaque Index[d]	Gingival Index[d]	Probing Depth[d]	BANA[e]
Whole-mouth	**r = 0.472**[f]	**0.404**	**0.375**	**0.383**	**0.536**	**0.581**	**0.402**
(Judge)	**p < 0.001**	**0.003**	**0.006**	**0.005**	**< 0.001**	**0.001**	**0.003**
Whole-mouth	-0.064	-0.068	-0.038	**0.283**	0.214	-0.070	0.049
(Self)	0.658	0.634	0.791	**0.042**	0.128	0.715	0.731
Tongue	**0.381**	**0.313**	**0.453**	**0.383**	**0.468**	**0.466**	**0.403**
(Judge)	**0.006**	**0.025**	**0.001**	**0.005**	**< 0.001**	**0.010**	**0.003**
Tongue	0.053	0.089	0.085	0.172	0.133	0.038	-0.058
(self)	0.711	0.535	0.549	0.223	0.346	0.841	0.685
Saliva	**0.439**	**0.413**	**0.477**	**0.433**	**0.583**	**0.513**	**0.463**
(odor judge)	**0.001**	**0.003**	**< 0.001**	**0.001**	**< 0.001**	**0.004**	**0.001**
Saliva	**0.385**	0.241	0.114	**0.321**	**0.402**	0.270	0.088
(self)	**0.005**	0.088	0.421	**0.020**	**0.003**	0.149	0.534
Preconception	-0.062	-0.049	0.011	-0.083	-0.086	-0.149	-0.121
(self)	0.668	0.738	0.942	0.563	0.547	0.432	0.398
Post-measurement	0.191	0.012	0.226	0.212	0.225	0.059	-0.056
(self)	0.179	0.936	0.107	0.131	0.108	0.756	0.692

[a,b] Log-transformed peak levels of volatile sulphide, measured intra-orally by means of a sulphide monitor (Rosenberg *et al.*, 1991a,b).
[c] Log-transformed cadaverine concentrations in saliva samples (Goldberg *et al.*, 1994).
[d] Mean index of whole mouth.
[e] Trypsin-like activity, based on average BANA scores of samples from four loci: shallow pocket, deep pocket, tongue dorsum, and saliva (Kozlovsky *et al.*, 1994).
[f] Significant correlations ($p \leq 0.05$) appear in bold.

depth, and trypsin-like activity), yielding r values ranging from 0.31 to 0.58 (corresponding p values ranging from 0.025 to < 0.001; Table 1).

In contrast, subjects' self-assessments showed few significant correlations with objective criteria. Preconception scores were not associated with volatile sulphide levels, laboratory or dental measurements, with r values ranging from -0.121 to 0.011 ($p \geq 0.4$). Subsequent self-assessments of whole mouth and tongue malodor did not (with the marginal exception of plaque index *vs.* whole mouth self-assessment, p = 0.04; Table 1) exhibit any significant associations with volatile sulphides, other laboratory parameters, or dental indices.

Among the five self-assessments, self-estimation of saliva was unique in its association with objective parameters. Saliva self-assessment scores were significantly associated with peak volatile sulphide levels (r = 0.39, p = 0.005), plaque index (r = 0.32, p = 0.02), and gingival index (r = 0.40, r = 0.003).

Although the post-measurement self-estimates were scored directly following saliva self-estimation, post-measurement scores were highly correlated with preconception and tongue self-assessments (r = 0.72 and 0.67, respectively, p < 0.001; Table 2) and were not significantly associated with clinical and laboratory measurements (p > 0.1; Table 1).

The five self-assessments and three odor judge assessments are compared in Table 2. Preconception scores were unrelated to odor judge scores (p > 0.25). Subsequent self-estimates of mouth and tongue malodor, as well as post-measurement scores, were not related to any of the odor judge scores, but were associated with preconception scores (r values of 0.49, 0.56, and 0.72, respectively, p < 0.001). Among the self-estimates, only saliva self-measurement was significantly associated with corresponding odor judge scores (r = 0.32, p = 0.023). Significantly, saliva self-estimates were also significantly associated with preconception scores (0.32; p = 0.023). When stepwise forward multiple-regression analysis of saliva self-assessment was carried out as a function of both odor judge saliva scores and preconception levels, both variables factored significantly (p = 0.02 for each parameter) in accounting for saliva self-scores, yielding a multiple r of 0.48 (p < 0.001). Subsequent post-measurement scores were highly associated with preconception (r = 0.72, p < 0.001) and unrelated to odor judge scores (p > 0.35; Table 2).

Table 3 compares the mean scores of self-assessments and odor judge scores. Differences in self- *vs.* odor judge assessments of whole mouth and saliva were not significant. In the case of tongue malodor, mean self-scores were more than twice as high as odor judge scores (means of 5.6 *vs.* 2.5 cm, respectively, p < 0.0001), and were similar to the mean

Table 2. Pearson analysis comparing judge and self-assessments with one another

	Whole-mouth (judge)	Tongue (judge)	Saliva (judge)	Precon-ception	Whole-mouth (self)	Tongue (self)	Saliva (self)
Tongue (Judge)	**0.725**[a] **< 0.001**						
Saliva (Judge)	**0.730** **< 0.001**	**0.599** **< 0.001**					
Preconception (Self)	-0.163 0.255	0.018 0.901	-0.113 0.431				
Whole-mouth (Self)	-0.159 0.261	0.069 0.629	-0.176 0.211	**0.488** **< 0.001**			
Tongue (Self)	-0.059 0.684	0.144 0.309	-0.063 0.657	**0.556** **< 0.001**	**0.716** **< 0.001**		
Saliva (Self)	0.260 0.062	0.223 0.113	**0.315** **0.023**	**0.319** **0.023**	**0.275** **0.048**	0.206 0.142	
Post-measurement (Self)	0.051 0.721	0.126 0.375	-0.023 0.874	**0.721** **< 0.001**	**0.574** **< 0.001**	**0.674** **< 0.001**	**0.495** **< 0.001**

[a] Significant correlations ($p \leq 0.05$) appear in bold.

levels of preconception and post-measurement self-assessments. Subjects and odor judge were also asked to score a foul odourant sample (fertilizer in a sniff bottle). Subjects' scores were closely clustered (8.8 ± 1.1 cm), were similar to the score given by the odor judge (8.2 cm), and were not correlated with any of the other parameters in the study.

The possibility that psychopathological symptoms may be linked to subjective self-reporting was tested with the SCL-90 questionnaire. Comparison of psychopathological profile with self-assessments is summarized in Table 4. Among the self-assessments, tongue odor and post-measurement scores were most closely associated with psychopathological parameters. Tongue self-assessments were significantly associated with somatization, depression, anxiety, paranoid ideation, and psychoticism categories, as well as with the general symptomatic index score. Post-measurement scores were significantly associated with somatization, depression, anxiety, phobic anxiety, paranoid ideation, and psychoticism categories, as well as with the general symptomatic index and positive distress index scores. In contrast, preconception scores were significantly associated with only two subscales, and neither whole mouth nor saliva self-assessments correlated with any of the SCL-90 parameters. No significant correlations were found between odor judge tongue scores and any of the psychopathological scales (data not shown).

Table 3. Comparison of mean self-assessment and odor judge scores[a]

Parameter	Self-assessment	Judge	Significance[b]
Preconception score	5.9 ± 2.8	-	-
Whole-mouth	3.5 ± 2.9	4.2 ± 1.9	0.137
Tongue	5.6 ± 2.8	2.5 ± 1.3	0.0001
Saliva	3.8 ± 3.1	3.4 ± 2.5	0.347
Post-measurement score	6.1 ± 3.0	-	-
Foul odor control[c]	8.8 ± 1.1	8.2	-

[a] Mean ± standard deviation of scores (in cm).
[b] Paired *t* test.
[c] Chicken-dung fertilizer in aqueous suspension.

Discussion

Although there is anecdotal and indirect evidence suggesting that people have trouble estimating their own bad breath (Iwakura *et al.*, 1964; Spouge, 1964), to our knowledge this is the first quantitative study to address this question directly. The results demonstrate that the subjects studied were generally incapable of scoring their own oral malodors in an objective fashion. Subjects' preconception scores, recorded prior to self-measurement, were not associated with the scores of the odor judge, the laboratory tests, or the dental measurements. Self-estimates of whole mouth and tongue malodor were closely related to preconception scores, and were similarly subjective. Only in the case of saliva were subjects partially capable of objective self-estimation. Nevertheless, in the subsequent post-measurement self-assessment, participants reverted to subjective scores closely resembling their initial preconception.

Table 4. Pearson correlations comparing subjects' self-assessments with their SCL-90 psychopathology scores[a]

Category	Preconception	Whole mouth	Tongue	Saliva	Post-measurement
Subscales					
Somatization	**r = 0.287**[b]	0.195	**0.265**	0.163	**0.244**
	p = 0.025[c]	*0.092*	***0.034***	*0.134*	***0.047***
Obsession-Compulsion	0.092	0.185	0.211	0.026	0.217
	0.269	*0.104*	*0.075*	*0.431*	*0.070*
Interpersonal Sensitivity	0.058	0.228	0.209	0.005	0.234
	0.350	*0.060*	*0.077*	*0.487*	*0.055*
Depression	0.095	0.238	**0.328**	-0.083	**0.270**
	0.263	*0.052*	***0.011***	*0.287*	***0.032***
Anxiety	0.158	0.165	**0.298**	0.068	**0.321**
	0.144	*0.132*	***0.020***	*0.324*	***0.013***
Hostility	0.124	0.012	0.150	0.165	0.218
	0.204	*0.469*	*0.155*	*0.131*	*0.068*
Phobic Anxiety	**0.258**	0.200	0.172	0.142	**0.288**
	0.040	*0.087*	*0.121*	*0.167*	***0.024***
Paranoid Ideation	0.040	0.223	**0.317**	-0.023	0.199
	0.395	*0.064*	***0.014***	*0.438*	*0.087*
Psychoticism	0.095	0.229	**0.362**	-0.042	**0.246**
	0.262	*0.059*	***0.006***	*0.388*	***0.046***
Global Scores					
General Symptomatic Index	0.131	0.218	**0.311**	0.033	**0.289**
	0.191	*0.068*	***0.016***	*0.412*	***0.023***
Positive Symptom Total	0.070	0.211	0.236	0.051	0.214
	0.320	*0.075*	*0.054*	*0.367*	*0.072*
Positive Distress Index	0.136	0.112	0.204	0.098	**0.278**
	0.181	*0.225*	*0.082*	*0.255*	***0.028***

[a] Among the 52 participants in the study, 48 filled out the SCL-90 questionnaire.
[b] Significant correlations ($p \leq 0.05$) appear in bold lettering.
[c] Numbers in italics represent significance levels.

The difficulty of sensing one's own bad breath has been previously attributed to adaptation (habituation) (Spouge, 1964; Engen, 1982). While the data do not rule out a role for adaptation in preventing objective self-assessment, self-measurement scores were not significantly lower than corresponding judge scores, as would be expected if adaptation were involved. Rather, the data suggest that subjects scored their own malodor largely according to erroneous, persistent, preconceived notions.

In other studies assessing self-estimation of body characteristics, subjects often exhibited a surprising inability to gauge their own body characteristics (Fisher, 1986). Schneiderman (1956) showed that, despite the familiarity of one's own face, recognition of its correct proportions is subject to considerable error. Several potential factors leading to exaggerated self-estimation of bad breath have been considered (Rosenberg and Leib, 1995). Advertisements on the subject of bad breath may elicit unwarranted concerns in suggestible people. Others may notice a bad taste in their mouth and erroneously assume that it must be related to bad breath. Children of parents with bad breath may grow up inferring that they also suffer

from the problem. Some subjects recall having been told only once in their distant past that they had bad breath, yet continue to worry about it.

The relatively high overall level of tongue self-assessment and its association with psychopathological traits, as determined by the SCL-90 questionnaire, are worthy of further investigation. One possible explanation is that the presence of foul odor on the wrist (which might otherwise be associated with pleasant perfume odors) evokes surprise or disgust associated with contamination or defilement. The high post-measurement scores and their relationship with the psychopathological parameters may derive from increased attention focusing on oneself as a result of the previous measurements.

The relative objectivity found in saliva self-scoring may be related to the more impersonal presentation of the odorant in a Petri dish, outside the body proper. This may be analogous to successful self-identification of body odor on T shirts which were removed prior to being smelled (Russell, 1976). Conversely, tongue odor self-assessment appears to be more closely related to preconceived notions and psychopathological factors. These two modes of self-examination for oral malodor (saliva and tongue) appear to accentuate objective and subjective stimuli, respectively, and thus may be of potential use in clinical assessment and diagnosis.

As in most other investigations of oral malodor, the majority of subjects in the present report (43) complained of bad breath (*i.e.*, either someone had told them they had bad breath, they thought they did, or both). Although it is likely that most adolescents and adults worry about bad breath (Yaegaki *et al.*, 1989; Scully *et al.*, 1994), experiments should be carried out to extrapolate these findings to larger populations of subjects who do not present with complaints of bad breath.

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DNA from *Serratia marcescens* confers a hydrophobic character in *Escherichia coli*

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Abstract

In order to determine whether hydrophobic surface properties of *Serratia marcescens* can be transferred to *Escherichia coli*, *E. coli* DH5α cells were transformed by DNA fragments from *S. marcescens* RZ. Fifteen-hundred *E. coli* transformants were screened for adhesion to hexadecane and polystyrene. One transformant exhibited increased adhesion to hexadecane droplets, as well as altered kinetics of aggregation in the presence of ammonium sulfate. Western colony blotting revealed that antibodies raised against *S. marcescens* RZ recognized component(s) on the transformant outer surface.

Keywords: *Serratia marcescens*; Hydrophobin; Cell surface hydrophobicity; Hexadecane; Ammonium sulfate aggregation; *Escherichia coli*

1. Introduction

The physicochemical outer surface properties of *Escherichia coli* have importance both from basic and applied viewpoints. Surface hydrophobicity and retention of *E. coli* cells on solid surfaces are a function of cell cycle as well as of the substratum surface energy [1]. In biotechnology, hydrophobized *E. coli* cells may be more readily immobilized than normal cells. Cell surface hydrophobicity may also play a role in adhesion of *E. coli* to host surfaces.

Compared to many other microorganisms, most strains of *E. coli* do not adhere readily to hydrophobic surfaces, such as hydrocarbons and plastics [7,8,14,17]. In the present study, we tested the feasibility of increasing cell surface hydrophobicity of a commonly used *E. coli* strain, following transformation with DNA from *Serratia marcescens*, a microorganism with pronounced adhesion to hydrophobic substrata [16]. *S. marcescens* was reported to possess hydrophobic surface properties by Mudd and Mudd 70 years ago [11,12]. Since then, cell surface hydrophobicity has been linked to partitioning of *S. marcescens* at air/water and oil/water interfaces, as well as adhesion to solid surfaces including catheters and other plastics [2]. Hydrophobic surface properties of *S. marcescens* appear to be mediated by

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several hydrophobins, including prodigiosin, a lipophilic tripyrrole pigment [3,5,6,15], and a 70-kDa surface protein, serraphobin [4].

2. Materials and methods

S. marcescens RZ was originally obtained from R. Zack, Tel Aviv University. Mutant 3164, a non-hydrophobic mutant, is derived from strain RZ [13]. Bacteria were maintained on brain heart infusion (BHI) agar (Difco Laboratories, Detroit, MI) at 4°C and transferred monthly. *E. coli* DH5α competent cells (library efficiency), were obtained from Gibco (BRL, Middlesex, UK) and were maintained at −70°C.

All molecular biology and cloning procedures used in this study were essentially as described by Maniatis et al. [10]. Chromosomal DNA was extracted from *S. marcescens* RZ and dissolved to yield a concentration of 0.25 mg ml^{-1}. DNA was digested with either *Bam*HI (New England Biolabs, Beverley, MA) or *Sau*3A (Biolabs). The *Bam*HI digest was selected for further treatments because it yielded a larger amount of DNA fragments of the desired size (5–10 kb) as compared to the *Sau*3A digest.

The *Bam*HI digest was fractionated by sedimentation through a sucrose gradient (5–20%), and the appropriate DNA fractions (i.e. size of 5–10 kb as determined by agarose gel electrophoresis), were pooled for ligation. The DNA fractions were ligated with T_4 DNA ligase (Biolabs) to *Bam*HI-cleaved pUC19 DNA (BioRad, Richmond, CA). Following $CaCl_2$ transformation of the ligation mixture into *E. coli* DH5α competent cells, transformants were selected on Luria Bertani (LB) plates (1% Tryptone, 0.5% yeast extract, 2.0% agar (Difco) 1% (w/v) NaCl), containing 50 μg per ml ampicillin (Sigma, St. Louis, MO) and 50 μg per ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Sigma, St. Louis, MO)).

White colonies which were ampicillin resistant were transferred again to the same plates and screened for hydrophobicity by testing the adhesion of the bacteria to liquid hexadecane as follows: to a 0.5-ml colony suspension in phosphate buffer (PB) containing 22.2 g $K_2HPO_4 \cdot 3H_20$, 7.26 g KH_2PO_4 per litre, pH 7.1, 0.1 ml hexadecane (Sigma) was added and the mixture was mixed vigorously for 1 min. Following phase separation, a sample (10 μl) of the upper hexadecane phase was taken for microscopic observation of bacteria adhering to the hexadecane droplets.

Measurement of the percent adhesion of the bacteria to hexadecane was performed as described previously [17]. Briefly, to 1.0 ml bacteria suspended to 1.0 OD in PB in a polystyrene cuvette, 0.1 ml hexadecane (Sigma) was added. The mixture was vortexed vigorously for 1 min. Following phase separation, the decrease in OD of the lower aqueous phase was measured in a spectrophotometer and the percentage of cells adhering to the hydrocarbon upper phase was determined.

Antiserum against whole cells of *S. marcescens* RZ was raised in rabbits as follows: 2×10^9 cells were injected i.v. three times with 3 days intervals between injections. 10 days following the last injection, blood was collected and antiserum was precipitated in the presence of 40% ammonium sulfate and was stored at −20°C. Additional blood collections were performed 3 days following a booster injection of 10^9 cells; blood samples were treated similarly.

The kinetics of ammonium sulfate-mediated aggregation was measured as follows: cells were inoculated 1:200 and grown overnight at 37°C in 50 ml of brain heart infusion broth (Difco). Bacteria were harvested by centrifugation and washed twice in PBS and suspended in 0.1 M ammonium sulfate to yield initial optical densities between 1 and 4.5 (400 nm, 1 cm light path). Aggregation over time was monitored by the decrease in optical density.

Anti-RZ antibodies, precipitated in 40% ammonium sulfate, were adsorbed onto non-hydrophobic mutant 3164 cells, as well as onto *E. coli* DH5α recipient cells harboring pUC19. For adsorption, cultures of *S. marcescens* 3164 were grown in 100 ml BHI for 24 h at 30°C. Following growth, cells were washed three times in 0.15 M NaCl by 20 min centrifugation at $10\,000 \times g$. The washed pellet was resuspended in 2 ml of the above antiserum preparation and the suspension was incubated with gentle shaking for 3 h at 4°C. The cells were then centrifuged as above and the supernatant was used to suspend a fresh washed cell pellet of strain 3164.

The procedure was repeated 3–6 times until sufficient specificity was obtained.

Western colony blotting was performed as described by Maniatis et al. [10]. Blots were incubated with the adsorbed anti-RZ antiserum.

3. Results and discussion

Among about 1500 clones possessing recombinant pUC19 plasmids with inserts of serratial DNA, one transformant, DH5α (pRB255), was found to exhibit adhesion to hexadecane ($31 \pm 23\%$, mean $\pm$ SD) which was significantly higher ($P < 0.005$, *t*-test) than that of control cells with the plasmid alone ($1.3 \pm 3.26\%$); wild-type hydrophobic donor strain *S. marcescens* RZ showed high adhesion to hexadecane ($78 \pm 5\%$, mean $\pm$ SD). The transformant was maintained on LB-amp plates (LB plates, containing 50 μg ml^{-1} ampicillin) at 4°C.

Restriction mapping of recombinant plasmid pRB255 revealed that it contained an insert of 5.4 kb (Fig. 1). In addition to the restriction sites depicted in Fig. 1, the insert contained four unresolved sites for *Sph*I, three for *Mlu*I, three for *Msc*I, three for *Bst*EII, and two for *Pst*I. The insert contained no sites for the enzymes *Hin*dIII, *Eco*RI, *Sca*I, *Xho*I, and *Kpn*I.

Ammonium sulfate has been proposed to aggregate *E. coli* cells, based on enhancement of hydrophobic interactions [9,14]. The kinetics of ammonium sulfate-mediated aggregation of adherent transformant DH5α (pRB255) and non-adherent recipient strain DH5α were compared at two initial cell densities (Fig. 2). At an initial optical density of 1, kinetics of aggregation were similar. However, when the initial cell density was raised to an OD of 5, different kinetics were observed. As compared to a constant decrease in optical density of the recipient strain, an S-type curve was observed for the transformant, with no aggregation during the first 100 min, followed by an extremely rapid decrease to low levels. In contrast, the recipient exhibited gradual

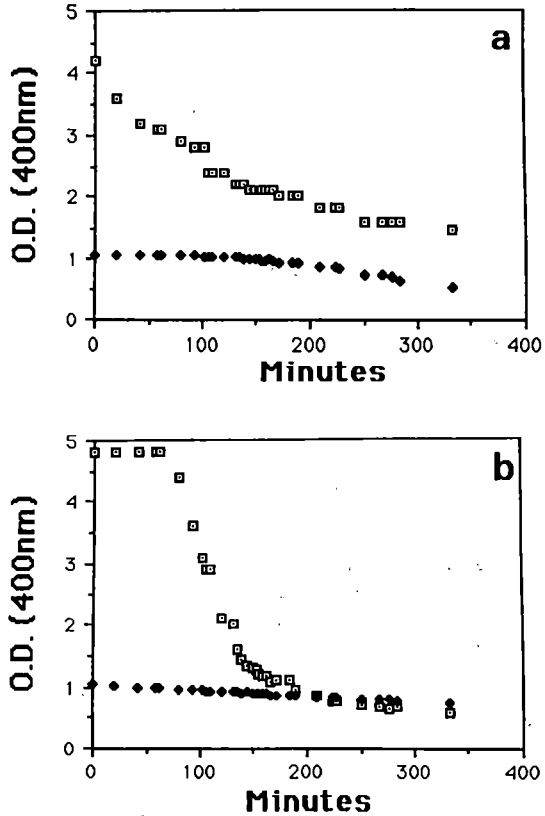

Fig. 2. Kinetics of ammonium sulfate-mediated aggregation of recipient non-adherent strain DH5α (**a**) and adherent transformant DH5α (pRB255) (**b**). Cells were washed and suspended in 0.1 M ammonium sulfate as described in Materials and methods and the decrease in optical density at 400 nm (1 cm light path) was measured over time at two initial optical densities (approx. 1 (♦) and approx. 4–5 (□)).

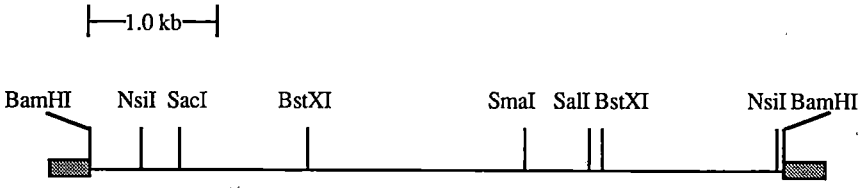

Fig. 1. Restriction map of recombinant plasmid pRB255. pRB255 DNA was cleaved with appropriate combinations of the depicted restriction enzymes in order to construct the restriction map. Restriction fragments were separated on 0.6% agarose gels and visualized following ethidium bromide staining. pUC19 sequences are represented by shaded boxes.

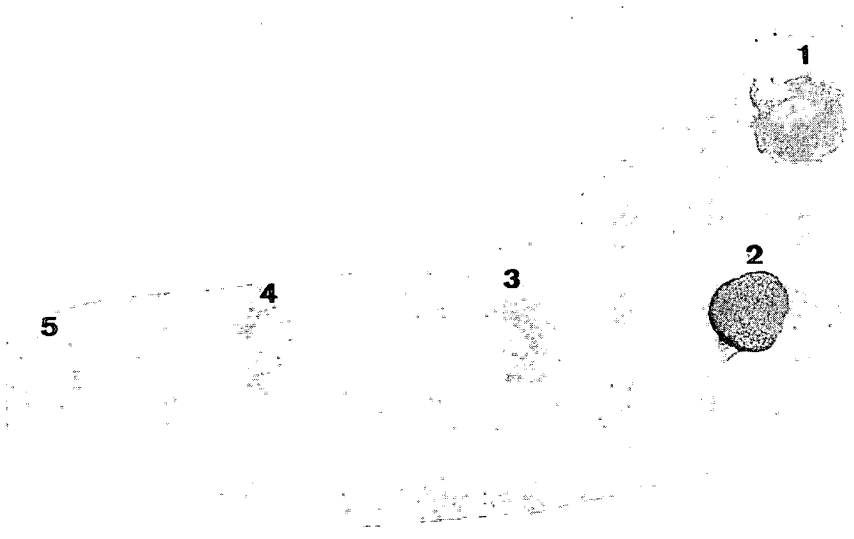

Fig. 3. Western colony blotting of the adherent transformant DH5α (pRB255). A colony of the adherent transformant DH5α (pRB255) as well as control reference colonies of RZ, non-hydrophobic strain 3164, and DH5α with or without pUC19 plasmid, were blotted as described in Materials and methods. Blots were reacted with anti-RZ antiserum which was pre-adsorbed on 3164 and DH5α cells. Right to left: 1, adherent transformant DH5α (pRB255); 2, RZ; 3, 3164; 4, DH5α; 5, DH5α (pUC19).

aggregation throughout the experiment (Fig. 2). The density-dependent aggregation in the transformant suggests a positive cooperative interaction between the cells. Furthermore, aggregation in both strains was more pronounced at the higher optical density. The dramatic difference in the aggregation kinetics of the transformant provides further evidence of its altered physicochemical properties.

Adherent transformant DH5α (pRB255) was examined by Western colony blotting (Fig. 3). A clear reaction with the anti-RZ antiserum was obtained with transformant cells and RZ cells, whereas the controls (DH5α with or without plasmid pUC19, and the non-hydrophobic mutant 3164) showed only a weak background reaction. Hence, we infer that the increased adhesion of the transformant to hexadecane, as well as the altered kinetics of aggregation in the presence of ammonium sulfate, are due to appearance of a *S. marcescens* antigen(s) on the surface of the transformed *E. coli* cells.

The present study supports the rationale of rendering *E. coli* cells more hydrophobic by transformation with DNA from other, more hydrophobic species. The results presented here extend previous data showing that *E. coli* cells can be hydrophobized by chemical surface modification [7] as well as by addition of organic cations [8]. Since these other treatments may affect viability, hydrophobization by genetic transformation may prove advantageous (e.g. in immobilization) to other methods which have an adverse affect on cell viability. This prospect is currently under investigation.

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Adhesion of Microbial Pathogens

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[44] Measurement of Microbial Adhesion to Hydrophobic Substrata

By RON J. DOYLE and MEL ROSENBERG

Introduction

Seven decades ago Mudd and Mudd[1,2] observed that some bacteria have a tendency to partition at oil–water interfaces. Their observations ultimately led to studies on the role of microbial adhesion in hydrocarbon degradation, the role of cell surface hydrophobicity in mucosal adhesion and pathogenesis, and the role of hydrophobicity in biofouling. The hydrophobic properties of microorganisms are known to contribute to their adhesion to various surfaces, including catheters, implants, teeth, red cells, mucosal cells, and plants. Hydrophobicity is best defined as the greater tendency of a microorganism to adhere to hydrocarbon or nonpolar substrata (hydrophobins) than to water. Numerous microbial pathogens seem to adhere totally or partially via the hydrophobic effect. These include members of the genera

[1] S. Mudd and E. B. H. Mudd, *J. Exp. Med.* 40, 633 (1924).
[2] S. Mudd and E. B. H. Mudd, *J. Exp. Med.* 40, 647 (1924).

METHODS IN ENZYMOLOGY, VOL. 253

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TABLE I

METHODS COMMONLY EMPLOYED TO MEASURE MICROBIAL CELL SURFACE HYDROPHOBICITY AND ADHESION TO HYDROPHOBINS

Method	Features
Microbial adhesion to hydrocarbon (MATH)	Microorganisms may adhere to hydrocarbon–aqueous interface; removal of cells from aqueous phase can be quantitated by spectrophotometry
Hydrophobic-interaction chromatography (HIC)	Octyl-Sepharose columns serve to trap hydrophobic microorganisms, whereas nonhydrophobic variants or strains do not adhere
Salt aggregation test (SAT)	Hydrophobic microorganisms, when mixed with ammonium sulfate, tend to aggregate; rates of aggregation can be monitored by turbidity readings
Contact angle measurements (CAM)	When water droplets are placed on dried lawns of microorganisms, the droplets form contact angles which are approximately proportional to surface hydrophobicity
Two-phase partitioning (TPP)	When microorganisms are mixed with two immiscible aqueous phases containing dextran and polyethylene glycol (PEG), hydrophilic cells tend to bind to the lower dextran phase, whereas hydrophobic cells partition into the more hydrophobic PEG upper phase
Direct binding	Small molecules, such as dodecanoic acid, can bind to hydrophobic cells, the binding of which can be determined by radioactivity assays
Adhesion to hydrophobic microspheres	Some hydrophobic microorganisms bind to polysytrene microspheres, the binding of which can be determined by light microscopy
Imprints of colonies	Microbial colonies, when pressed gently on the surface of polystyrene, are lifted off the agar substratum; washing the polystyrene leaves an "imprint" of the hydrophobic colonies

Bacillus (spores), *Candida, Escherichia, Staphylococcus,* and *Streptococcus* among others. A general review of microbial cell surface hydrophobicity can be found in Doyle and Rosenberg.[3] Although there are exceptions, microorganisms which have a high cell surface hydrophobicity exhibit a high tendency to adhere to hydrocarbons.

There are now numerous methods to measure microbial adhesion to hydrophobins and cell surface hydrophobicity. Each appears to have certain advantages. It is unlikely a single method will satisfy all requirements, however. Table I offers an outline of the most commonly used methods. Usually, the methods correlate quite well when care has been taken to

[3] R. J. Doyle and M. Rosenberg (eds.), "Microbial Cell Surface Hydrophobicity." American Society for Microbiology, Washington, D.C., 1990.

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employ common buffers, cells of the same age, constant temperature, consistent levels of salts, etc. When salt concentrations are too low, microbial suspensions frequently do not readily bind hydrocarbons or plastics. In addition, cold room temperatures (2°–5°) give much lower binding to hydrophobic surfaces or molecules than temperatures in the 30°–40° range. Experience with the systems described in Table I suggests that optimal measurements of hydrophobicity are made at pH 5–6 and at least 0.2 *M* sodium chloride and 37°. The following section details use of the microbial adhesion to hydrocarbon (MATH) assay.

Microbial Adhesion to Hydrocarbon

The MATH assay is based on the tendency of certain microorganisms to adhere to the surfaces of liquid hydrocarbon droplets, during a brief mixing period.[4,5,6,7]

Utensils

Glassware should be clean and preferably acid-washed, as surfactants and other impurities can interfere with results. Disposable plastic pipettes and cuvettes can be used in most instances (one notable exception being use of a test hydrocarbon which is incompatible, e.g., *p*-xylene).

Preparation of Microorganisms

In general, microorganisms which have been grown in liquid medium are employed in the MATH assay. However, in certain cases cells may be scraped from agar plates. In other cases (e.g., dental plaque, feces), cells may be taken directly from their sources. Following microbial growth or direct isolation, cells are washed at least twice in buffer. The recommended buffer is 50 m*M* acetate containing 200 m*M* sodium chloride (pH 5.6). This buffer is often replaced by phosphate buffer (50 m*M* phosphate buffer plus 150 m*M* sodium chloride, pH 7.2). The assay may be attempted in other buffers, but ionic strength and pH may have an effect on results obtained. Following washing, the optical density of the microbial suspension is usually adjusted to approximately 0.8–1.0 at 450 nm. This is arbitrary, and the results obtained may depend on the initial cell density employed.

[4] M. Rosenberg and R. J. Doyle, *In* "Microbial Cell Surface Hydrophobicity" (R. J. Doyle and M. Rosenberg, eds.), p. 1. American Society for Microbiology, Washington, D.C., 1990.
[5] M. Rosenberg, *FEMS Microbiol. Lett.* **22**, 289 (1984).
[6] M. Rosenberg, *FEMS Microbiol. Lett.* **25**, 41 (1984).
[7] M. Rosenberg, M. Barki, R. Bar-Ness, S. Goldberg, and R. J. Doyle, *Biofouling* **4**, 121 (1991).

Test Hydrocarbon

In the original assay, three test hydrocarbons were chosen for the assay: *n*-hexadecane, *n*-octane, and *p*-xylene. In subsequent studies, hexadecane was used more frequently, although other test hydrocarbons sometimes provide important results which are not evident employing hexadecane. Hexadecane, obtained commercially, may on occasion contain contaminants. For example, hexadecane may be oxidized to yield components which can interfere with the assay. Hexadecane can be protected from oxidation by storage under nitrogen gas. A simple procedure was reported for determining whether the hydrocarbon and other assay components are relatively surfactant-free. If the buffer and test hydrocarbon are mixed together (with no microbial suspension) under the assay conditions and quickly coalesce following the mixing procedure, no significant contamination is evident. If, however, droplets do not coalesce quickly, or an emulsion is formed, then an interfering contaminant may be present. It is convenient to mix hexadecane with a mixed-bed ion-exchange resin to remove interfering components. The resin MB-3 is ideal for such use. Care must be taken when using aromatic hydrocarbons, because of possible toxicity to humans, damage to the microbial cell wall and membrane, and incompatibility with polystyrene and other plastics.

Assay

The original assay was performed with 1.2 ml microbial suspensions, in test tubes overlaid with samples of test hydrocarbon ranging from 0.01 to 0.2 ml. Obviously, volume differences as well as differences in the ratio of the aqueous and hydrocarbon phases affect results. The test tubes containing the microbial suspension and test hydrocarbon are vortexed for a set 2-min period. Following vortexing, the phases are allowed to separate. If no adhesion takes place, the phases should coalesce and separate completely. In the case of adhesion, a "creamy" upper layer is formed, consisting of cell-coated hydrocarbon droplets. Adhesion should always be confirmed by microscopic observation of the droplets. Adhesion is most readily quantified by measuring the drop in turbidity in the aqueous phase following the mixing procedure. The lower aqueous phase may be withdrawn using a Pasteur pipette and transferred to a cuvette for an optical density measurement. However, the assay can be performed directly within a cuvette or tube that is compatible with a spectrophotometer, in which case the decrease in turbidity can be determined directly, provided that (i) the hydrocarbon droplets have risen completely from the aqueous phase; (ii) care is taken to ensure that the upper phase does not interfere with the light beam; and

(iii) the walls of the vessel are free of interfering droplets (they can usually be detached by shaking the vessel once or twice sharply by hand).

The decrease in optical density should always be compared with that of the suspension prior to mixing. It is erroneous to incorporate as a control a vortexed test tube or cuvette with no hydrocarbon, because in the absence of the test hydrocarbon microorganisms may attach in large numbers to the walls of the glass or plastic. It is also unacceptable to measure turbidity following solidification of the liquid hydrocarbon phase at low temperatures, because this causes desorption of the cells from the oil–water interface. Microorganisms which do not adhere to hydrocarbons under one set of conditions cannot be inferred as being "nonadherent." Rather, several attempts should be made at altering various aspects of the assay (pH, type of hydrocarbon, etc.).

Kinetic Approach

Based on the observation that adhesion to hydrocarbons is time-dependent,[8] several investigators have developed kinetic approaches in which adhesion to different hydrocarbon volumes is followed as a function of time. This is most readily performed in 4-ml cuvettes, containing 1 ml of aqueous phase and hydrocarbon volumes ranging from approximately 5 to 50 μl, and taking time points of several seconds. Following each brief vortex, the phases are allowed to separate, the turbidity of the lower phase measured, and the mixture subjected to further mixing/measurement cycles. For a given aqueous-to-hydrocarbon volume ratio, the decrease in turbidity over time follows pseudo-first-order kinetics, yielding a removal rate. Plotting the removal rates as a function of the hydrocarbon-to-water volume ratio usually yields a straight line, the slope of which is termed the "removal coefficient."

Several aspects of the kinetic analysis require further clarification. For example, back extrapolation of the removal rate to zero hydrocarbon volume yields a finite number, rather than zero adhesion rate as one would predict. Furthermore, the removal coefficient may be a function of the initial cell density, suggesting some type of cooperative interaction between the cells and the hydrocarbon. Figure 1 shows a set of data for a typical MATH assay.

Modifications

Addition of various compounds may have a profound effect on MATH. For example, the presence of low concentrations of amphipathic cationic

[8] D. Lichtenberg, M. Rosenberg, N. Scharfman, and I. Ofek, *J. Microbiol. Methods* 4, 141 (1985).

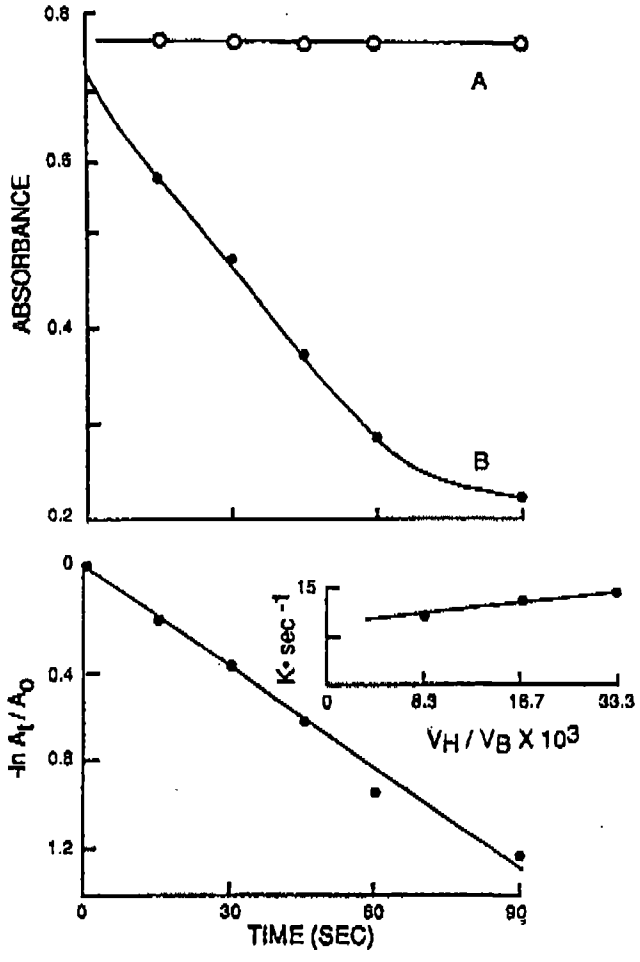

FIG. 1. Adhesion of *Streptococcus cricetus* AHT and *Bacillus subtilis* 168 to hexadecane. (*Top*) Curve A shows adhesion of *B. subtilis* to the hydrocarbon as a function of mixing time; curve B, adhesion of *S. cricetus* to hexadecane. (*Bottom*) First-order rate plot for the binding of *S. cricetus* to hexadecane, using data derived from the top curve. (*Inset*) The slope of the first-order rate plot (K min^{-1}) versus the ratio of hexadecane to buffer (V_H and V_B, respectively). The volume of cells in buffer was 3.0 ml for curves A and B (top), whereas the amount of hexadecane was 100 μl. The results show that *B. subtilis* 168 did not exhibit any tendency to adhere to the hydrocarbon, whereas *S. cricetus* was highly adherent. Independent assays have shown that *B. subtilis* has no ability to bind plastics or other hydrophobic surfaces.

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agents, such as cetylpyridinium chloride and chlorhexidine, as well as cationic biopolymers [lysozyme, chitosan, poly(L-lysine)] can potentiate microbial adhesion to hydrocarbons.[9] The assay can also be modified for enrichment of mutants with altered surface properties. Microorganisms bound to hexadecane droplets can serve as a substratum for subsequent attachment of coadhering partners.[10]

Advantages and Disadvantages

Advantages of the MATH assay include (i) low cost of disposable items and materials, and ubiquitousness of instrumentation (vortex, photometer); (ii) its rapid nature, both in operation and in generation of data; (iii) the ability to observe the adhering cells by light microscopy; and (iv) measurement of the adhesion characteristics of the entire microbial suspension, rather than a small fraction of cells which usually comes in contact with a given surface.

The main disadvantages of the assay are that (i) the surface of attachment is not constant, precluding steady-state analysis; (ii) assay conditions may vary from laboratory to laboratory, making it difficult to compare results; and (iii) some bacteria autoagglutinate, making spectrophotometric readings unreliable.

Salt Aggregation Test

The salt aggregation-test (SAT), like the MATH assay, is inherently simple and requires no specialized equipment.[11,12] Basically, a suspension of bacteria is mixed with an equal volume of an ammonium sulfate (AMS) solution and observed for aggregation. The AMS solutions usually are 1–4 *M*, but could be lower if the bacteria are highly hydrophobic. In some cases, the AMS can cause the bacteria (or spores) to adhere avidly to polystyrene wells, the adhesion of which can be quantitated by enzyme-linked immunosorbent assay (ELISA)-like assays. In one case, very low densities of spores of *Bacillus anthracis* were quantitated by use of AMS-promoted adhesion of the spores to plastic surfaces. The spores were assayed on the plates by use of soybean agglutinin conjugated to horseradish

[9] S. Goldberg, R. J. Doyle, and M. Rosenberg, *J. Bacteriol.* **172,** 5650 (1990).
[10] H. C. Van der Mei, M. Rosenberg, and H. J. Busscher, *in* "Microbial Cell Surface Analysis" (N. Mozes, P. S. Handley, H. T. Busscher, and P. G. Rouxhet, eds.), p. 263. VCH Publ., Brooklyn, New York, 1991.
[11] C. J. Smyth, P. Johnsson, E. Olsson, O. Soderlind, J. Rosengren, S. Hjertén, and T. Wadström, *Infect. Immun.* **22,** 462 (1978).
[12] M. Lindahl, A. Faris, T. Wadström, and S. Hjertén, *Biochim. Biophys. Acta* **677,** 471 (1991).

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peroxidase (ELLA, or lectinosorbent assay).[13] In other studies, employing more dense suspensions of microorganisms, it has been found convenient to measure the rate (as determined by plots governing first-order kinetics) of aggregation induced by AMS. If AMS concentrations are too high, the cells may flocculate, thereby rendering rate assays useless. When AMS concentrations are too low, aggregation may occur, but there could be a significant lag time before the rate of aggregation follows first-order kinetics. Usually, hydrophobic organisms yield much shorter lag times than nonhydropohobic variants.

Contact Angles and Cellular Hydrophobicity

Van der Mei *et al.*[10] and Busscher *et al.*[14] have reviewed the procedures needed to obtain reproducible contact angles. Basically, when a droplet of water is placed on a hydrophobic surface (a lawn of bacteria), the contact angle is proportional to the hydrophobicity of the surface. For bacteria, water contact angles are usually made by use of a system to determine the change in angle as a function of time. A requirement for contact angle measurements is that the surface must be dry. A further compounding factor is that the droplet may rapidly hydrate a partially hydrophobic bacterial lawn. In spite of these difficulties, the method clearly distinguishes between bacterial lawns, and it is useful in studying the differences in hydrophobicity between certain pathogens and avirulent mutants. Contact angles do not, of course, measure adhesion to hydrocarbons. Nevertheless, contact angles are useful in predicting adhesion of certain pathogens to hydrophobic surfaces.

Hydrophobic-Interaction Chromatography

Hydrophobic-interaction chromatography (HIC) is useful in separating hydrophobic from hydrophilic organisms and in estimating the proportion of a population capable of adhering to hydrocarbons. Agarose beads conjugated with phenyl or octyl groups are commercially available. The beads are washed with phosphate-buffered saline (PBS) or acetate buffer and poured into a column (Pasteur pipettes plugged with glass wool make convenient columns). A small volume of cells is poured onto the column and eluted with buffer. Fractions are then collected for analysis. It is conve-

[13] K. Graham, K. F. Keller, J. Ezzell, and R. J. Doyle, *Eur. J. Clin. Microbiol.* 3, 210 (1984).
[14] H. J. Busscher, J. Sjollema, and H. C. Van der Mei, *in* "Microbial Cell Surface Hydrophobicity" (R. J. Doyle and M. Rosenberg, eds.), p. 335. American Society for Microbiology, Washington, D.C., 1990.

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nient to grow cells in the presence of a tritiated amino acid mix or tritiated thymidine in order to obtain radioactive suspensions. Conversely, the fractions may be diluted and assayed for viable counts on suitable agar plates. An alternative and rapid method involves the suspension of cells with the conjugated beads. The beads are allowed to settle and samples of the supernatant assayed for viable counts, radioactivity, turbidity, etc.

The HIC method does not work well when bacteria capable of binding galactose are employed, as agarose contains galactosyl groups. Members of the genera *Pseudomonas, Escherichia,* and *Streptococcus* have been reported to bind galactose. In some cases, cross-linked dextrans (Sephadex) may be substituted for agarose. In addition, another control is usually run employing a nonconjugated agarose. This control is useful in assessing the trapping of cells in the bead suspension. Finally, the HIC method is not useful when cells tend to autoagglutinate. In some cases cell aggregates (as well as chains found in streptococci) may be disrupted by mild sonication. Sonication, however, may have an effect on surface hydrophobins.

Conclusion

The foregoing summarizes the method most commonly employed in determining adhesion to hydrophobins. In general, there is a good correlation between MATH and the other assays.[4] For this reason and the fact that the method is simple and reproducible, MATH has become the assay of choice for many laboratories. There are numerous factors involved in adhesion to hydrophobins, such as pH, ionic strength, temperature, age of cells, growth medium for the cells, time of incubation (or vortexing), antibiotics, and others.

Acknowledgments

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ריחות מהפה — איבחון וטיפול

מל רוזנברג־נבו[1,2],
ימוריס וגבריאלה גולדשלאגר, ביה"ס לרפואת שיניים, הפקולטה לרפואה סאקלר, אוניברסיטת תל־אביב; [2]והמרכז הרפואי מרום בזל, תל־אביב

ריח רע מהפה (האליטוזיס) הוא מצב שכיח הקשור לתחומים רבים, בין היתר רפואה, רפואת שיניים, מיקרוביולוגיה, ביוכימיה, כימיה ופסיכולוגיה. בניגוד לדעה הרווחת בציבור, ריחות פה ניתנים בד"כ לאיבחון ובמקרים רבים גם לטיפול [1-4]. במאמר זה ננסה לתת מענה למספר שאלות הקשורות לאיבחון וטיפול בריח רע מהפה, ובנוסף לכך נעלה מספר שאלות, שלא הסתמן בהן פיתרון עדיין, לגבי נושא מעניין זה.

האחריות לאיבחון של ריחות פה והטיפול בהם מוטלת בראש ובראשונה על ציבור רופאי השיניים, וזאת מכיוון שברוב המכריע של המקרים, ריחות רעים מהפה נובעים מפעילות חיידקים בתוך חלל הפה עצמו [1-4]. כמו־כן, ריח רע מהפה שמקורו בחלל הפה, ניתן בד"כ לטיפול בקלות יחסית, באמצעות הדרכה נאותה וטיפול שיניים שיגרתי, או כמעט שיגרתי. במקרים בהם הריח הרע אינו נובע מהפה, על רופאי השיניים להפנות את המטופל לרופא מתאים להמשך הטיפול [1].

ריח רע מהפה עצמו

החוקרים בדעה, כי לרוב המבו..רים וכן לילדים רבים בעיית ריח רע מהפה מורגשת באופן כרוני, או בזמנים מסוימים במשך היום. כמו־כן, ברוב המכריע של המקרים (90% היא הערכה סבירה לדעתי) הריח הרע נובע מהפה עצמו [1-6]. החיידקים המצויים בחלל הפה הם האחראים לכך. הם מייצרים גזים בעלי ריח דוחה, בראש ובראשונה H_2S ומתילמרקפטן, בעיקר כתוצרי לוואי של חילוף חומרים בתגאים אנאֶרוביים [7]. לפיכך, ריח רע מהפה יכול לנבוע ממספר מקומות בחלל הפה, בהם מסתתרים חיידקים, מקומות אשר לא תמיד מגיעים אליהם באמצעות שיטות ניקוי שונות. סיבות לריח־רע מהפה עצמו כוללות: ניקיון לקוי של הפה, מחלות חניכיים, יובש בפה, אוכל שנתקע בין השיניים, תיקוני שיניים שנעשו לא כראוי, תותבות לא נקיות, פעילות מופרזת של חיידקים על גב הלשון, מורסות ו־oral carcinomas [1-4].

ריחות פה אופייניים

בתוך חלל הפה ניתן להבחין בכמה סוגי ריחות שונים, המרמזים על מקור הריח:

א' ריח של רובד על־חניכי — ריח זה איננו דוחה במיוחד, ומצוי לדוגמה בילדים קטנים, שאינם מצחצחים שיניים.

ב' ריח של רובד תת־חניכי — ריח זה, שהוא חריף במיוחד, אופייני לפעילות חיידקית בין השיניים ובתוך הכיסים המתפתחים בין השיניים לחניכיים. ניתן לדגום ריח זה ע"י העברת חוט דנטלי בכיס דקתי, או בין שתי שיניים צמודות, שלא זכו לניקוי כגון זה זמן רב. גם שאריות בשר שניתפסו בין השיניים מדיפות ריח דומה. כמו־כן, ריח של tonsilloliths יכול לעיתים להזכיר ריח זה, אולם תרומתם הכללית של השקדיים לריח הפה טרם נחקרה ביסודיות.

ג' ריח מהחלק הקידמי של גב הלשון (anterior tongue dorsum) — החלק הקידמי של גב הלשון הוא לרוב בעל ריח חמצמץ. ניתן לדגום ריח זה ע"י ליקוק פרק כף היד והרחתו כעבור מספר שניות. ריח זה מפתיע בעוצמתו, אך מניסיוננו תרומתו לריח הפה הכללי איננה גדולה.

ד' ריח מהחלק האחורי של גב הלשון (posterior tongue dorsum) — זה שנים רבות ידוע אזור זה כאחד המקורות החשובים והיותר שכיחים להיווצרותו של ריח פה [4]. הוצע לדגום את המקום עם פד גאזה [8], אך מניסיוננו החומר נספג, ולא מושג ריח מייצג. בשנתיים האחרונות, הננו מגרדים אזור זה בכפית פלסטיק, כדי לקבל דגימה לתצפית והרחה. מצאנו, כי ריח חזק מלווה בד"כ בהפרשה צהובה־ירקרקה או חומה, המזכירה זו שבנזלת. אך לעומת נזלת רגילה, שהיא נטולת ריח, חומר צמיג זה הוא בעל ריח דוחה ואופייני. השערתנו, אשר טרם אושרה מדעית היא, שחומר זה מקורו ב־"postnasal drip". כיוון שגב הלשון שופע חיידקים, הריר שנדבק ללשון עובר תהליך של ריקבון, היוצר את הריח המיוחד.

ה' ריח של תותבות — ריח זה מתאפיין אף הוא בתכונות מיוחדות, בהיותו בעל יסוד מתקתק אך דוחה. הריח אופייני לתותבות שאינן מוצאות במשך הלילה לשרייה, או שאינן מנוקות היטב.

ו' ריח מדרכי האף — גם ריח שמקורו בדרכי האף הוא בעל אופי משלו, המזכיר במקצת גבינות צרפתיות מסוימות. בספרות ההאליטוזיס הוא מכונה בטעות "ozena" [9]. לאנשים רבים ריח אף חולף, שתלוי כנראה באלרגיות או במזג האויר. דלקת גיתות ועצמים זרים באף (במיוחד אצל ילדים בעלי פיגור שיכלי) עלולים לגרום לריח אף חריף. אולם, קיימים מקרים רבים של ריח אף כרוני שאינם מלווים במימצאים פתולוגיים כלשהם. קיימת השערה כי התייבשות הריריות בדרכי האף יכולה לגרום לריח זה. ייתכן שגם rhinoplasty יכולה להוות במקרים מסוימים גורם לריח אף.

ז' ריחות פה ממקורות אחרים — בשנת 1982 ערכו Attia & Marshall [3] רשימה ארוכה של מחלות, שיכולות לגרום לריח רע מהפה. בין אלה מצוינות מחלות קשות, ואף קטלניות, כגון עגבת, סוגי סרטן מסוימים וכשל תיפקוד הכבד. לחלקם ריח מאוד אופייני, כגון הריח המזכיר ריח דגים, הקשור לכשל תיפקוד מערכת הכליות. החומרה הפוטנציאלית של מצבים כגון אלה, ממחישה את חשיבות הפניית המטופל לבדיקה רפואית מתאימה באופן מיידי.

בניגוד לדעה הרווחת בקרב הציבור, ריח פה כרוני הנובע מה־

קיבה נדיר ביותר, היות שבמצב רגיל הוושט סגור (collapsed) ומונע דליפת גזים [4].

ריח רע מדומה

מסיבות שאינן ברורות עדיין, אנשים על־פי־רוב אינם מודעים לריחות הנובעים מפיהם. כך קורה פעמים רבות שאנשים סובלים במשך שנים מריח רע מהפה, מבלי להיות מודעים לכך, עד שבני משפחתם הקרובה או חבריהם הטובים מעירים את תשומת ליבם לכך. קיים גם מצב הפוך, שבו אנשים מדמיינים, כי ריח הפה שלהם הרבה יותר חזק ודוחה ממה שהוא באמת. הסיבות לריח פה מדומה אינן ברורות. ייתכן, שהדבר קשור למיבנה האישיות של הפונה, ולרגישותו ביחס לתדמיתו בעיני הזולת. בחלק מהמקרים, נזכר המטופל בבן משפחה (בד"כ אב או אם) הסובלים מהבעיה, ומשליך מכך לגבי עצמו. בספרות הפסיכיאטרית ריח פה מדומה מוגדר כ־delusion, אך יש מעט מאוד מחקרים בנושא זה. במקרים רבים אנשים הסבורים כי הם סובלים מריח רע מהפה, בין אם הדבר מבוסס ובין אם לאו, מתרחקים מהחברה, ולעיתים אף שוקלים להתאבד.

איבחון ריח רע מהפה

האם יש לבוא בטענות כלפי הרופאים ורופאי השיניים על כך שאינם מייחסים תשומת־לב מספקת לקיומו של ריח רע מפיותיהם של המטופלים שלהם? למעשה, המטופלים עצמם מקשים לעיתים קרובות על האיבחון, בכך שהם מצחצחים את שיניהם ומשתמשים בתרסיסים ובתכשירים מבושמים זמן קצר ביותר לפני הבדיקה. הדבר מסווה ומוחק באופן זמני את מדד האיבחון החשוב הזה. על הרופא לפיכך, לעמוד על־כך שהמטופלים המתלוננים על בעיה זו יצמצמו לכדי מינימום פעולות בחלל פיהם (כלומר, יימנעו מאכילה, שתיה, עישון, ציחצוח, לעיסת מסטיק, מציצת סוכריות, שימוש בתרסיס או שטיפה במי־פה) לפחות שעתיים לפני הביקור. אם למטופל נודע מבן משפחה קרוב (אשתו למשל) כי הוא סובל מריח רע מהפה, על בן־משפחה זה להיות מוזמן לבדיקה. הוא יוכל לספק נתונים חשובים, אשר יאמתו את התלונה, יסייעו לבדיקת מקור הריח, ויוכל אף לדווח על שיפור שיחול עקב הייצרץ והטיפול. את ריח הפה של המטופל יש לאבחן בכמה צורות. תחילה מתבקש המטופל לנשוף דרך פיו; אולם, לעיתים, עוצמת הריח חלשה בעת הנשיפה, אך חזקה הרבה יותר בעת הדיבור. על־כן פיתחנו בדיקה נוספת, לפיה מבקשים מהנבדק לספור עד 20 בקול רם, ובו זמנית מריחים את הבל פיו ("count to twenty" test). לאחר הרחת חלל הפה מבקשים מהמטופל לנשוף דרך האף ומריחים גם את ריח האף. ריח רע הנובע מהפה בלבד מרמז על־כך, שמקורו בחלל הפה. ריח רע הנובע בעיקר מהאף יכול להצביע על כך שהבעיה היא בגיתות האף. ריח רע זהה מהאף ומהפה יכול להעיד על סיבה מערכתית. לעיתים נובע הריח הרע בר־זמנית ממספר סיבות (לדוגמה, מחלת חניכיים, ריח מגב הלשון ובעיות בגיתות האף).

מטופלים הסובלים מריח פה רע, שמקורו בפה, חייבים להיבדק באופן יסודי. הבדיקה צריכה להקיף את כל המקומות האפשריים לפעילות מיקרוביאלית: יש צורך לברר האם המטופל סובל ממחלות חניכיים; מה מצב הנקיון בפה; האם קיימת הצטברות של אוכל או רובד חידקים בין השיניים, בצמוד לגשרים או בסחימות לקויות. האם המטופל סובל מחוסר רוק — באופן כרוני או רק לעיתים; האם לשונו של המטופל שעירה או לחלופין מכוסה בשיכבה עבה; האם יתכן, שהריח קשור לגרון; אם קיים חשש שהריח קשור בליחה, יש לבדוק זאת. אם הריח נובע מהאף, ניתן להמליץ על בדיקת אא"ג מדוקדקת, כולל אנדוסקופיה.

בחלק מן הנבדקים אף־על־פי שהריח בא מהפה, קשה להצביע על המקור המדויק. במקרים כאלה מכונה הריח "לא־פתולוגי" או "פיסיולוגי" [2] וניתן לייחס זאת לרמה כללית גבוהה של חידקים על הלשון הצמודים לרירית הפה, לרמת רוק נמוכה (כרונית או זמנית), וכד'. על־מנת לאפיין את המטופלים הסובלים מבעיה זו, פותחה בדיקה פשוטה (oratest) המספקת הערכה כמותית על רמת הפעילות החידקית בחלל הפה [10, 11]. הבדיקה כוללת שטיפת הפה בדגימת חלב עקרה, המועברת לתוך מבחנה המכילה אינדיקטור חימצון־חיזור (methylene blue). ככל שרמת המיקרואורגניזמים גבוהה יותר, כך משתנה הצבע מהר יותר מכחול (תנאים אֲרוביים) ללבן (תנאים אנאֲרוביים) בתחתית המבחנה. כונן מיוחד מאפשר לרופא ולמטופל לעקוב אחר שינויי הצבע כפונקציה של זמן. בנוסף למיתאם עם ספירות חידקים, תוצאות בדיקת ה־oratest משתנות באופן בולט כאשר מבצעים שטיפה במי־פה יעילים [12].

מטופלים הסובלים מריחות פה שאינם נובעים בבירור מחלל הפה או מגיתות האף, יש להפנות לבדיקות רפואיות כוללניות. ריחות כאלה יכולים לנבוע מבעיות במערכת הנשימה, מחלות סיסטמיות שונות, ושימוש בתרופות מסוימות. במקרים מסוימים, יכול ריח־פה רע להצביע על מחלה קשה. לפיכך, איבחון מהיר והפנייה מתאימה חיוניים.

לעיתים קרובות מתלוננים מטופלים על ריח־פה רע, למרות שהבודק אינו מריח דבר. ריח רע מהפה אכן מתחזק ונחלש במידה מסוימת במשך היום, ובד"כ מחריף בעוצמתו שעות מספר לאחר האכילה. כאשר מתעורר ספק אם אכן קיים ריח פה רע יש לחזור ולבדוק את המטופל ולנסות להתייצץ עם בני משפחתו, כדי לוודא האם קיים בכלל ריח פה. אם לאחר מספר בדיקות לא מתגלה ריח רע מהפה, ייתכן, שהמטופל סובל מריח רע מדומה. במקרים כאלה ניתן להמליץ על ייצרץ פסיכולוגי, אך מנסיוננו מעטים בלבד אכן פועלים על־פי עצה זו.

מדידה כמותית של ריחות פה

קיימות מספר דרכים לכימות מידת ריח רע מהפה. דרכים אלה מתבססות על רמות הגופרית המחזרת הנמצאות בהבל הפה [1, 2]. בשיתוף עם ד"ר יעקב גבאי, הראינו שמכשיר ניטור, שנועד לבדיקות סביבתיות של H_2S יכול לספק ערכים כמותיים המתאימים במובהקות של $p<.0001$ לתוצאות המתקבלות ע"י קבוצת שופטים [8, 14]. חשיבותו של כימות הריח טמונה בכך, שהוא מספק רמה התחלתית ומאפשר מעקב אחר שיפורים שחלים בעיקבות טיפול זה או אחר, ביחס לרמה זו [1]. לשימוש במכשיר מדידה כמותית נודעת חשיבות אף במחקרים קליניים [12]. מדידה כמותית של הריח אינה מסייעת באיתור מקור הריח, אך ייתכן שמיכשור כזה יפותח בעתיד.

הטיפול בריח רע מהפה

הטיפול בריח רע מהפה חייב להתחיל בפה עצמו. גם במקרים שריח רע מהפה אינו נובע מהפה עצמו, אלא מסיבה אחרת, ישנה לעיתים

תוספת של ריח רע מחלל הפה עצמו. במרבית המקרים גיהות פה נכונה חיונית להפחתת רמת הריח הרע מהפה [1-6]. זו כוללת טיפולי שיניים תקופתיים, שימת דגש מיוחד על מוקדים בכוח להתפתחות חידקים. היות שמחלות חניכיים הן גורם משמעותי לריח רע מהפה, ודי במוקד אחד כדי לגרום לריח רע, איבחון וטיפול מיידי עשויים לפתור את הבעיה.

מעבר לשימוש סטנדרטי במיברשת שיניים, ניתן להמליץ על שימוש בחוט דנטלי. רופאי שיניים מתלוננים לעיתים קרובות, שמטופלים רבים אינם משתמשים בחוט דנטלי. לדעתנו, מטופלים שיהיו מודעים לאפשרות, כי עלול להיות להם ריח רע מהפה בגלל גיהות פה ברמה נמוכה, יהיו מוכנים להשתמש באופן קבוע בחוט דנטלי. הרחת החוט עצמו ע"י המטופלים יכולה להועיל מאוד. אזורים בין השיניים המאופיינים בריח החזק ביותר, הם אלה שיש לנקותם בצורה הטובה ביותר. ציחצוח שיניים, כפי הנראה, אינו משפיע דיו על צימצום מיידי של ריח רע מהפה, שכן רבים מתלוננים על התגברותו מחדש של הריח הרע מהפה דקות ספורות לאחר הציחצוח. עם זאת, לציחצוח כפי הנראה השפעה לטווח־ארוך בשמירה על השיניים והחניכיים. ציחצוח הלשון, הכולל את חלקה האחורי, יכול להפחית את עוצמת הריח, בפרט כאשר ניתן לזהות את גב הלשון כמקור ריח בכוח.

שטיפת הפה

שטיפת הפה הטובה ביותר אינה נעשית בצורה מלאכותית־מסחרית (מי־פה), אלא על־ידי בלוטות הרוק שלנו. לעיתים קרובות קיים מיתאם בין רמות של ריח רע מהפה לירידה ברמת הרוק [13]. ריח רע בבוקר עם היקיצה, נובע מהצטברות חידקים ותוצרי הלוואי הנדיפים שלהם במשך הלילה, פרק זמן בו ייצור הרוק כמעט שנפסק לגמרי [7]. לפיכך, הגברת זרימת רוק, ע"י לעיסה למשל, תקטין בד"כ את עוצמת הריח הרע מהפה. ניתן להמליץ למטופלים ללעוס ירקות סיביים במשך היום, או מסטיק נטול־סוכר במשך דקות ספורות. רבים סבורים, ששטיפת הפה במי־ברז תורמת להורדת רמת ריח הפה. ייתכן שזה נכון, אך רק לטווח קצר. עם זאת, בשטיפת הפה במים נשטפים גם ציפויי רוק מגינים מעל המשטחים הרירים, ובכך המצב למעשה מחמיר.

מתי להמליץ על שימוש בשטיפות פה?

קיימת מעט מאוד ספרות מדעית שיכולה להנחות רופאים לגבי הה־שפעות לטווח ארוך של שטיפות במי־פה. במקרים מסוימים, טיפול שיניים נאות המלווה ברמה גבוהה של שמירה על נקיון הפה, יוריד באופן משמעותי את רמת הריח הרע מהפה. עם־זאת, רוב האנשים אינם מסוגלים לנקות את פיותיהם ביעילות גבוהה מספיק, והריח ממשיך להפריע. בנסיבות כאלה, ניתן לשקול שימוש במי־פה. רוב סוגי מי־הפה המיוצרים מסחרית יעילים הודות לגורמים נוגדי־חידקים, כגון צטילפרידיניום כלורידי (CPC), אלכוהול אתילי, אבץ כלורידי, שמנים אתריים וכלורהקסידין. במשך 12 השנים האחרונות, עסקה המעבדה שלנו בגישה חלופית, שהובילה לפיתוח תכשיר מי־פה דו־שיכבתי שמן: מים. מיקרואורגניזמים של הפה ותאים אפיתליים מתקלפים רבים נדבקים אל טיפות השמן הזעירות הנוצרות תוך כדי עירבוב, ועקב כך מסיר התכשיר מיקרואורגניזמים ולכלוך מחלל הפה. התכשיר מכיל CPC המשפר את עירבוב השמן והמים, מגביר את היצמדות החידקים לטיפות, ומעכב את פעילות החידקים הנותרים בפה לאחר הגירגור [14]. נמצא, כי לתכשיר מי־פה זה יש יכולת להפחית את רמת הריח הרע מהפה לפידקי זמן ארוכים יחסית. מי־פה המכילים כלורהקסידין יעילים אף הם בהפחתת רמת הריח הרע מהפה, אך אינם מומלצים לשימוש מעבר ל־2-3 שבועות [12].

למי־הפה, ככל הנראה, השפעה מרבית כאשר השימוש בהם נעשה לפני השינה. בתנאים כאלה גורמים מעכבי־חידקים הקיימים בתכשירי מי־הפה שוהים בפה למשך פירקי זמן ארוכים יותר, ויכולים לסייע בניטרול השפעת הירידה בזרימת הרוק במשך הלילה.

מי־פה יכולים אף לסייע באיבחון הראשוני של מקור הריח הרע מהפה. ירידה בריח הרע מהפה בעיקבות שימוש במשך מספר ימים בתכשיר מי־פה אפקטיבי, היא סימן טוב לכך, שלחלל הפה מעורבות ביצירת הריח הרע [8].

שאלות בלתי פתורות

שאלות רבות הקשורות לנושא של ריח רע מהפה לא נדונו דיין ע"י קהילית החוקרים. אחת השאלות הסבוכות ביותר היא "מדוע אנשים בעלי ריח רע מהפה אינם מודעים לריחם הרע?" תשובה אפשרית אחת לשאלה זו היא "הרגל". אנשים מתרגלים לריח מסוים במשך פרק זמן, עד שאינם יכולים עוד לחוש בו. עם זאת נמצא, שמטופלים מסוגלים בד"כ להריח ריח שנלקח מלשונם או ריח מחוט דנטלי בו השתמשו, ולעיתים יכולים אף להריח את ריח הפה שלהם ע"י כיסוי האף והפה בידיים והרחת הבל פיהם. אולם, האם זו רמת הריח שחשים אנשים אחרים מסביב? בימים אלו נערך מחקר המנסה לענות על שאלות אלה.

שאלות רבות אחרות נותרו למחקר בעתיד: האם ריח רע הוא דבר סובייקטיבי המשתנה מתרבות לתרבות? האם הוא מושפע מאקלים? האם יכול ריח־פה לשמש כמבחן איבחוני אמין למחלות חניכיים? האם מולקולות גופריתיות הן המרכיב היחיד האחראי לריח רע מהפה, או שגורמי ריח נוספים תורמים לכך? [15].

לסיכום, ריח רע מהפה הינו בעיה המשותפת לאנשים רבים. על הרופאים ורופאי השיניים מוטלת החובה להתריע בפני המטופלים על האפשרות שהם סובלים מריח רע מפיהם. מטופלים המתלוננים על ריח רע מהפה צריכים להיבדק בדקדקנות וביסודיות, ורצוי שיעשה שימוש במדדים כמותיים. לעיתים קרובות נקיון פה לקוי ובעיות דנטליות אחרות הן הן הגורמות לריח רע מהפה. על בעיות אלו ניתן באופן כללי להתגבר ע"י טיפול שיניים נאות, שמירה על נקיון הפה, ניקוי גב הלשון ושימוש במי־פה יעילים. במקרים אחרים יש לערוך בדיקות רפואיות מתאימות על־מנת לגלות את מקור הבעיה.

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תזונה וחומצות אמיניות בתיפקוד נשימתי

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מח' לטיפול נמרץ נשימתי, ביה"ח רמב"ם, חיפה

חסי הגומלין שבין תזונה לבין תיפקוד נשימתי מוכרים היטב. הפחתה בכמות הקאלוריות גורמת ירידה בקצב חילוף החומרים ולהפחתה באיוורור הריאתי [1, 2]. מאידך גיסא, תזונה גורמת לחילוף חומרים מוגבר ועשויה אף לגרום לגירוי הנשימה [3].

חולים הלוקים בנַפַחַת הריאות (אמפיזמה) או בסמפנת כרונית, מצויים לעיתים קרובות במצב של תת־תזונה [4]. בחולים אלה, נקשרת הירידה במשקל בעלייה בתיצרוכת האנרגטית במנוחה (תאב"מ)* ובשיעור תמותה גבוה יותר [5-8]. לאחרונה הצטבר מידע על הקשר בין כמות החלבונים בברות לבין התיפקוד הנשימתי.

מטרתנו בסקירה הנוכחית לבחון את השפעת המנגנונים לפעולת חומצות אמיניות על מרכז הנשימה, ועל תיפקוד השרירים (ובעיקר שרירי הנשימה), תוך ציון השלכות קליניות לשימוש בחומצות אלה. כל זאת נבדק במחלות נשימה שונות, כגון תיסמונת דום־נשימה בשינה, אל־נשימה בפגות, עייפות שרירים או ככלי עזר בשלבי גמילה מהנשמה מלאכותית.

השפעות תת־תזונה חלבונית על מערכת הנשימה

שניים עד שלושה ימי־צום גורמים בבעלי־חיים לירידה במשקל, בכוח שרירי הטרפש וברמות הגלוקוזה בדם [8, 9], למרות שמשקלו של הטרפש, עוביו ותכולת הגליקוגן שבו, נותרו ללא שינוי. המחברים מניחים, שמצב היפוגליקמיה הוא הגורם להפחתה ביכולת התכווצות הטרפש, הנצפית בצום קצר־מועד.

רעב ממושך (3 שבועות), גרם להפחתה בתכונות האלסטיות של ריקמת שרירי הטרפש בעכברים [10]. חסר תזונתי נפרץ במצבים של מחלות ריאה, ומצוי עד כדי 50% מבין החולים במחלות נשימתיות חדות [11]. Rogers וחב' [12] הראו, שבכ־70% מהחולים במחלות ריאה חסימתיות כרוניות (מרח"כ)* שהתקבלו לאישפוז, אירעה ירידה במשקל.

אם ניתן להפיק תועלת מהפחתה במשקל בחולי מרח"כ עם משקל־יתר (בעיקבות הפחתה בצורכי חילוף החומרים המובילה להפחתה בדרישות הנשימתית), הרי שאילו חולים דומים, בעלי משקל תקין, ירדו במשקלם ניתן יהיה לראות החמרה בתיפקוד הנשימתי [13].

הוכח, שעלייה בתיצרוכת האנרגטית במנוחה, הנצפית בחולי מרח"כ עם תת־תזונה, היא הגורמת לאובדן המשקל. העלייה בתיצרוכת האנרגטית במנוחה נגרמת קרוב לוודאי עקב הגברת פעולת הנשימה [14].

Arora ו־Rochester [15] הראו, ששרירי הנשימה אינם נשמרים מהתהליך הקאטבולי המאפיין שרירים אחרים בגוף במצבי רעב ממושך או במצבי דחק קטאבוליים. הירידה בנפח שרירי הטרפש מקבילה, לדעתם, לזו הנצפית בשרירי השלד האחרים, אך ההפחתה הנצפית בתיפקודם עולה על זו שהיתה צפויה עקב אובדן הנפח בלבד. ייתכן, שהסיבה נעוצה באספקת גליקוגן ירודה או בשינויים ביוכימיים של תאי השריר. מסקנה זו מתחייבת מאחר שהזנה מחודשת של חולי מרח"כ גורמת לשיפור בתיפקוד שרירי הנשימה זמן רב לפני שהובחן בשרירים אחרים בגוף [16]. חולים בתת־תזונה ללא מחלה ריאתית מדגימים הפחתה בכוח שרירי הנשימה [17]. הפחתה זו עולה באופן ישיר למידת הירידה במשקל.

הנשמה מלאכותית, המציינת מצב של כישלון נשימתי, משפיעה באופן משמעותי על שרירי הנשימה תוך גרימת ניוון.

מצד שני, ניתן לראות בהנשמה מלאכותית, גורם המסייע להפחתת עבודת שרירי הנשימה, ירידה בתיצרוכת החמצן, הדרושה כל כך במצב שיחלוף הגזים הפגום. מיד לכשמשתפר התיפקוד הנשימתי, מן הצורך להתחיל בתהליך הגמילה מההנשמה. נראה.

* תאב"מ — תיצרוכת אנרגטית במנוחה.

* מרח"כ — מחלות ריאה חסימתיות כרוניות.

Diagnosis and Treatment of Bad Breath

Recent progress in understanding the underlying scientific principles governing oral malodor

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Bad breath is a common human condition. References to the medical significance of bad breath and methods for its treatment date back several thousand years.[9] Most of us probably suffer from bad breath at least occasionally.[39] From a commercial point of view, concern over bad breath spawns a multi-billion-dollar business. Most sales of mouthwashes and mouth-freshening sprays result from concern over breath freshness,[17] as do significant portions of sales in several other categories, including chewing gum and mint candies.

Oral malodor research has garnered interest recently, starting with a symposium held in Mexico in 1991 under the auspices of the International Association for Dental Research. This was followed, in 1993, by the First International Workshop on Oral Malodor, in Israel. *Bad Breath:Research Perspectives,* based on the workshop, was published last year.[23] The Second International Workshop on Oral Malodor, held in Leuven, Belgium, October 1995, drew 130 interested researchers and clinicians from around the world.

What Causes Bad Breath?

Most cases of bad breath (85% is a rough estimate) originate in the oral cavity. As confirmation of this assessment, oral rinsing with any liquid usually reduces odor levels immediately.[17,39] Some otolaryngologists believe the tonsils to be a common source of bad breath,[6] but this has not been confirmed. Malodor can also originate in the nasal passages, quickly identified by smelling the air exiting a person's nostrils. Among nonoral etiologies of bad breath, nasal malodor predominates.

Contrary to popular belief, bad breath from the stomach is exceptionally rare (except of course, as the result of belching).[38] Metabolic disorders may also produce bad breath; the fish odor due to trimethylaminuria is an example. And, while a wide variety of diseases, such as abcesses of the respiratory tract, carcinomas or liver diseases, can lead to bad breath, all these contribute to only a very small fraction (less than 2% in my estimation) of cases.[2,21]

Oral sources of malodors: Within the oral cavity, an important source of oral malodor is the gumline, particularly in the proximal regions between the teeth where bacteria accumulate. In most (but not all)[3] clinical studies to date, subjects with periodontal disease had higher levels of malodor than did the healthy cohorts. Subjects with periodontal disease also have greater accumulations of microorganisms, debris and VSC on their tongues compared with healthy subjects.[39] By simply smelling the dental floss (or contoured

Malgré l'intéret général tres répandu au sujet de la mauvaise haleine, on a dirigé peu d'efforts à comprendre les principes scientifiques fondamentaux. Cet étude résume le progrès récents dans ce domaine et discute quelques considérations commerciales pertinentes.

Trotz des weitverbreiteten allgemeinen Interesses an Mundgeruch sind relativ kleine Anstrengungen unternommen worden, die entsprechenden wissenschaftlichen Prinzipien zu verstehen. Dieser Bericht fasst neue Erkenntnisse auf diesem Gebiet zusammen und bespricht mehrere kommerzielle Betrachtungen.

A pesar del interés general difundido en maloliento oral, relativamente poco esfuerzo se ha hecho en comprender los principios científicos subyacentes. Este informe es un resume del progreso reciente en esta área y discute varias consideraciones comerciales pertinentes.

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toothpick) after each pass between teeth, a person can both check the intensity of this very characteristic odor and locate its origin(s). Mechanical debridement, particularly tooth brushing and flossing, play a large role in maintaining fresh breath.

Figure 1-1. Several kinds of plastic tongue scrapers have been manufactured in North America and Europe, but their use is not widespread.

Another important oral source of bad breath is the tongue dorsum, a prime growth location for oral microorganisms.[3] Bad breath stemming from the tongue dorsum usually smells quite different from the odor coming from the gums. One simple way of apprising malodor from the tongue is simply to have your subject lick his or her wrist, wait several seconds, then take a sniff. However, the important odor source is usually farther back towards the throat.[12] This is best sampled by scraping with a plastic spoon.[25]

In many instances, a yellowish discharge is found. This discharge may actually consist of postnasal drip, which accumulates on the tongue and is degraded there.[41] Because of the mucous nature of this discharge, its position on the tongue and Western society's rather nonabrasive diet, mastication and swallowing do not seem to remove it. As with dental plaque, the treatment of choice appears to be mechanical debridement. Whereas there are several plastic instruments available dedicated to this purpose (Figure 1-1), a simple toothbrush usually suffices, provided its contour enables the user to clean the back of the tongue with minimal gagging.

Other oral sources of bad breath include inadequate dental treatment and oral hygiene, for instance, overhanging restorations, crown leakage, food impaction or incomplete scaling.[39] Saliva and mastication are important modulators of oral malodor. When saliva flow wanes, as it does overnight, during a lengthy conversation, as the result of medication or stress, or during fasting, malodor increases.[39]

Oral malodor in women may increase during menstruation, as the result of hormonal changes resulting in transient gingivitis.[35] Denture odor has its own characteristic smell.[25] Smoking causes another frequent and distinct breath odor, smoker's breath.[41]

What is Bad Breath?

Among the VSCs found in mouth samples by Tonzetich and coworkers, hydrogen sulfide and methyl mercaptan predominate, with minor contributions of dimethyl sulfide and others.[39] The contribution of nonsulfurous gases is at present unclear.

Several studies have implicated indole, skatole and methylamine as malodorous compounds in putrefied saliva, which might be present in mouth air.[7,15] In 1967, Tonzetich and coworkers showed that these compounds, when added to saliva, were not detectable in the headspace.[40] However, they neglected to consider the possibility that these components could become volatile when oral surfaces dry out; indeed, oral malodor worsens when the mouth is dry.

Kleinberg and Codipilly recently showed that a variety of malodorous components could be volatilized from saliva

A Short History of the Study of Bad Breath

Writings on oral malodor date back to Greek and Roman times.[8,22,35] Gum mastic, the resin obtained from the *Pistacia lentiscus* tree, has been chewed for thousands of years around the Mediterranean as a breath freshener; it may be the original chewing gum.

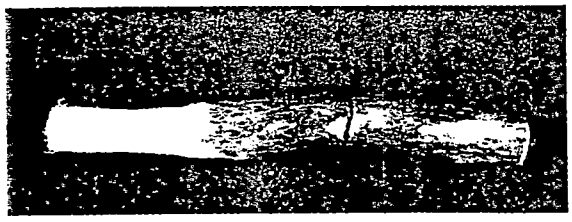


Figure 2-1. The "Siwak" is a special stick for cleansing the oral cavity. When the tip of the branch is soaked, fibers are formed, which can then be used to clean the teeth, gums and tongue.

Both Jewish and Islamic teachings deal at length with problems associated with bad breath.[24] The Jewish Talmud contains several mouthrinse formulations; faithful believers still concoct at least one of these, although not on a commercial basis. Islamic teaching stresses the use of a special wooden stick, the "Siwak," for cleaning the teeth and preventing bad breath (Figure 2-1).[24] Other ancient remedies include chewing cloves (Iraq), peppercorns (the Talmud) and parsley (Italy).

In 1874, Joseph Howe wrote the most recent single-authored, English-language book published on bad breath. By 1898, it had undergone at least four printings.[13] Observation and opinion served as the basis for most writings on bad breath until about 60 years ago. During the 1930s and 1940s, Fosdick and his colleagues established a scientific basis for oral malodor research using the osmoscope, an instrument enabling threshold detection of odors as a function of dilution.[36] These studies helped establish that microbial putrefaction within the oral cavity causes most bad breath.

The main scientific breakthrough to date came in the 1960s. Tonzetich and coworkers employed gas chromatographic techniques to establish an association between volatile sulfur components (VSCs) and oral malodor. VSCs can be used as indicators of bad breath levels.[39]

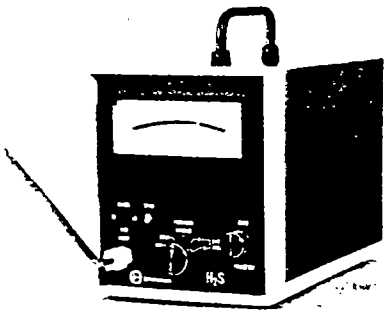


Figure 1-2. The sulfide monitor (model 1170, Interscan Corp., Chatsworth, CA), used to demonstrate an association between oral sulfide levels and bad breath scores assigned by odor judges. The inlet of the monitor is easily fitted with a disposable straw through which researchers can collect headspace samples directly from the mouth.

Self-Assessment of Oral Malodor

One of the more interesting paradoxes related to bad breath is that millions of people appear to suffer from it without ever noticing it, while millions of others harbor greatly exaggerated and unjustified fears of suffering from bad breath ("halitophobia").[25] This apparently stems from our inability to smell our own bad breath. Earlier researchers attributed this to adaptation to one's own oral malodor.[22,35] In a recent study, 52 individuals were asked to grade their own bad breath following a series of self-measurements. Indeed, subjects were generally unable to score their own malodor levels objectively.[4] Rather, self-scoring was intimately related to subjective, preconceived notions. Self-measurement of saliva odor gave the only objectivity attained.

dried on skin at the wrist and that the smell could be detected by an odor judge several minutes later.[14] Thus, the contributions of amines, aliphatic acids and other compounds to mouth odors need re-examination. Recently, we have significantly associated salivary levels of cadaverine with both bad breath and periodontal disease parameters.[9,10] Further work is needed to determine whether cadaverine can be detected in mouth air as well.

Bacterial Causes

Clearly, bacteria are responsible for bad breath originating in the oral cavity. The long-term reductions in malodor resulting from controlled rinsing with antimicrobial rinses, such as those containing 0.2% chlorhexidine gluconate, support this conclusion.[31] However, the precise nature of the microorganisms responsible for the various odors is unknown.

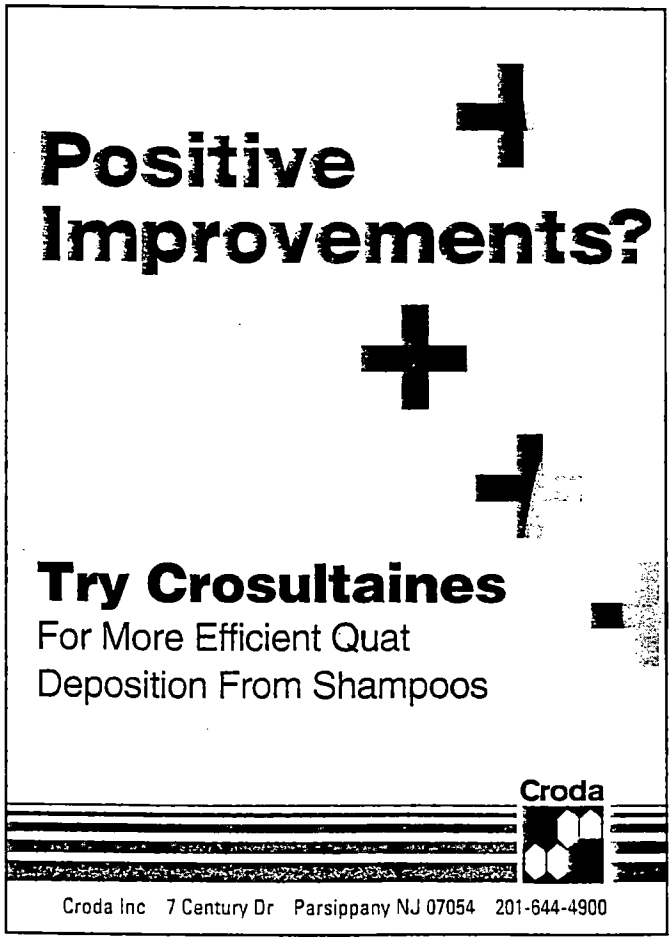
A large proportion of oral bacteria grown in vitro on serum produced hydrogen sulfide and methyl mercaptan.[20] Several studies suggest that anaerobic Gram-negative microbiota, such as *Fusobacterium, Treponema, Porphyromonas* and *Prevotella* species, produce oral malodors.[14]

Measuring Bad Breath

For many years, panels of odor judges measured bad breath. With the advent of gas chromatography, an instrumental assessment could be used in addition to or instead of sensory assessment.

My laboratory has developed protocols for using portable sulfide monitors to measure the oral presence of malodor-associated sulfides (Figure 1-2).[28,32,39] This instrument was previously used for other applications, such as atmospheric measurements of hydrogen sulfide and electrochemical factory monitoring, that require sensitive (ppb level), on-site, real-time measurements. Monitor results show a significant correlation ($p<0.001$) with the scores of a 7-member odor-judge panel.[28] Since our initial study, several oral malodor investigations have been published using the sulfide monitor.[3,24,32]

In general, the correlation coefficients comparing sulfide-monitor results with odor-judge measurements fall in the 0.45-0.65 range. Thus, whereas the correlations themselves are highly significant, they account for only about 20-40% of the variation of the odor-judge scores.[24] One possible reason for the moderate level of association is that, as previously mentioned, sulfides account for only one class of bad breath components. The advantages of measurement with the sulfide monitor include portability, immediate readings, rapid turn-around between measurements.



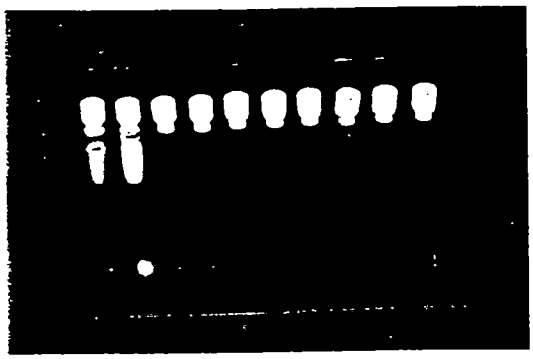


Figure 1-3. In the Oratest, individuals rinse their mouths with UHT sterilized milk, which is then transferred to a test tube containing methylene blue. The tubes are then placed in a rack over a mirror. Anaerobic respiration causes the liquid to change from blue (tube on left) to white (adjacent tube). The time required for the change correlates with the microbial levels present.

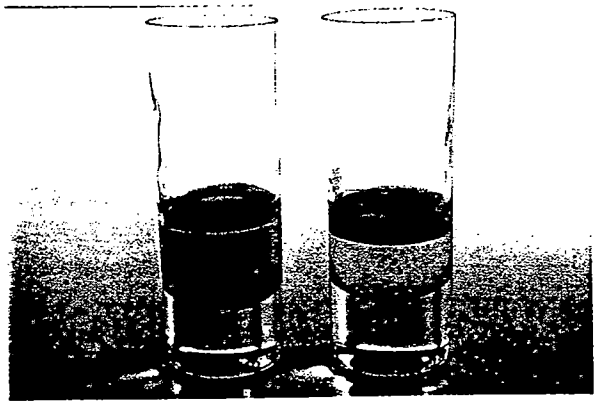


Figure 1-4. The two-phase mouthwash formulation, before (left) and following (right) use. The food coloring in the aqueous phase doubles as a biological stain. Following expectoration, the color has disappeared from the aqueous phase; it now appears on the droplets. Microscopic observation of the droplets reveals that they are covered by numerous microorganisms and oral debris.

noninvasiveness and low cost. The main disadvantage, as compared with gas chromatography, is that all sulfhydryl compounds are lumped together in one score. Furthermore, the instrument responds to high levels of alcohol and essential oils, disallowing its use for scoring breath sulfides immediately after using many mouthrinses.

In assessing the bad breath of individuals who complain of the problem, measurement of volatile sulfide levels can be used as a quantitative adjunct to, but not instead of, direct smelling of the air exiting the mouth and nose. The Halimeter, a sulfide monitor developed by Interscan Corp. for use in dental clinics, is finding a market in "fresh-breath dental clinics" across North America. However, dentists operating such services should appreciate the current limitations of instrumental analysis in assessing bad breath among patients.

The Oratest, another simple test developed in our laboratory, uses an oral rinse to assess oral microbial activity (Figure 1-3).[26,33] The Oratest is derived from the age-old assay for determining the level of microbial activity in milk samples by incubation in the presence of methylene blue, an indicator that loses its blue color under anaerobic conditions. In the original test, the time required for loss of the blue color during incubation was an indication of the microbial level and potability of the milk sample. We use the same color change to measure microbial activity in the oral cavity.

Methods: In the Oratest, an individual rinses the mouth vigorously with a sterile (UHT) milk sample for 30 seconds. The sample is then added to a test tube containing methylene blue and allowed to stand. Researchers, observing a mirror positioned under the tube, record the time required for the aerobic-to-anaerobic, blue-to-white change. Oratest scores correlate significantly with microbial counts,[33] as well as with clinical indices[37] and improvements in oral malodor levels.[27] In a recent pilot study of 24 children, the Oratest was the test most significantly associated with odor-judge scores of all the tests measured.[34]

Treating Bad Breath

Treatments for bad breath may be mechanical, chemical or a combination of both. Mechanical treatments recomended include toothbrushing, deep tongue brushing, interdental cleaning (using dental floss, anatomical toothpicks or interdental brushes), brief mastication (of fibrous vegetables or chewing gum) and periodic calculus removal.

Consumers most commonly use mouthrinses to chemically treat oral malodor,[1,19] although chewing gums and tablets may also act as treatment vehicles. Among the antibacterial agents used, chlorhexidine is probably one of the most, if not the most, effective.[27,32] Its wide antibacterial spectrum and substantivity in the mouth probably give the long-term reductions in oral malodor levels observed.[1] Although continuous use of chlorhexidine is discouraged, a chlorhexidine rinse can help the initial diagnosis of whether an individual's odor derives from the oral cavity.

Listerine,[a] a best-selling mouthwash containing essential oils and alcohol, provided long-lasting reduction in bad breath, when rinsed twice daily for 30 seconds over 6 weeks.[16] In actual practice, however, most consumers probably gargle with Listerine for just a few seconds.[1,19] Other mouthwashes reported to reduce bad breath include those containing zinc salts and quaternary ammonium compounds like cetylpyridinium chloride.[18]

Several oxygenating rinses, such as those using chlorine dioxide, are marketed as breath-freshening agents. However, few scientific data on efficacy are available. In addition, the question of potential carcinogenicity, due to the generation of chlorites, which can react with organic molecules,[5] has yet to be fully addressed.

Similarly, there is a trend toward reducing the concentrations of ethyl alcohol in mouthrinse formulations. Alcohol in mouthrinses adds "bite" to the product, enhances the flavor, solubilizes essential oils and has antibacterial activity.[1] However, there may be some adverse health considerations. Alcohol may actually exacerbate oral malodor with its astrin-

[a]Trademark of Warner-Lambert Co.

gent properties. Other potential effects not sufficiently examined include the possibility that alcohol is metabolized in the mouth to yield odorous compounds and that alcohol residues in the mouth "lift" malodorous components, in much the same way that alcohol in perfumes "lifts" the top notes. The above notwithstanding, the complete absence of alcohol from a mouthrinse formulation is not necessarily a guarantee of its subsequent commercial success.

Bacterial adhesion to oil droplets: My own interest in bad breath grew out of research on the adhesion of microorganisms to hydrophobic surfaces.[29] In the early 1980s, research in this laboratory showed that microorganisms scraped directly from the tooth surface adhere to oil droplets in high proportions.[30] Researchers subsequently found that oil droplets are similarly able to remove bacteria still attached to tooth surfaces.[31]

Work in this laboratory has focused on a proprietary two-phase mouthwash that combines mechanical and chemical approaches to treating bad breath (Figure 1-4). Adding cetylpyridinium chloride to the aqueous phase potentiates adhesion of oral microorganisms and debris to the oil droplets.[11,27] Besides the proven ability of oil droplets to bind and desorb microorganisms from surfaces,[29-31] adding essential oils and triclosan or other antibacterial agents to the oil phase boosts efficacy.

Assuta,[b] the two-phase mouthwash based on these principles introduced in the Israeli market in November, 1992, has become a successful commercial product (Figure 1-4).

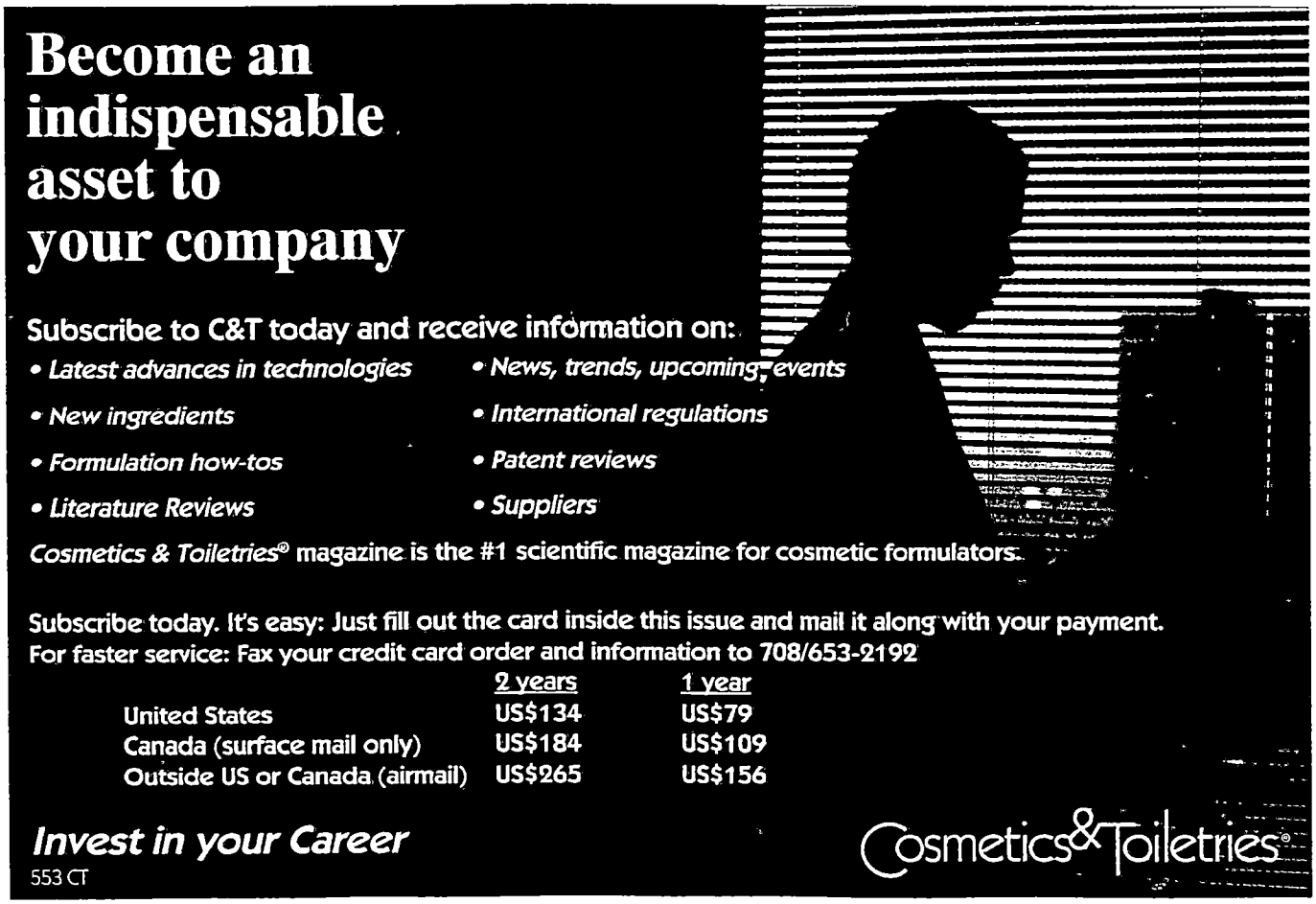
[b]Trademark of Shemen Soad Ltd.

In a 6-week study, Assuta outperformed Listerine in long-term (9- to 20-hour) reduction of whole-mouth odor, as well as odor deriving from the posterior tongue dorsum.[16]

Product Development and Testing

Recent research suggests that chemists consider the following points when developing, testing and marketing a mouthrinse or other oral product for breath-freshening purposes:

- Since oral malodor involves several types of odors, it is not advisable to use a single parameter in testing, as this may be misleading. For example, a reduction of 50% in volatile sulfides does not mean an equivalent reduction in odor judge assessments. Measurement should include several parameters, including odor judge scores of both whole mouth and tongue dorsum.
- Gargling of a mouthrinse toward the throat may be as important as, or more important than, rinsing the front of the mouth, especially in people whose odor derives from the back of the tongue.
- Current oral rinses, candies and sprays do not take the place of mechanical debridement — brushing teeth and tongue, flossing and similar measures.
- Because of the difficulties involved in smelling one's own oral malodor and the embarrassment involved in asking, individuals who use breath-freshening products often have little or no idea what works and what does not. Therefore, consumers often use taste and oral feel as cues for their assessment of bad breath reduction.
- Most mouthwashes do not work for a long time.[17] Thus,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a real need exists for potent mouthwashes with lasting effect and minimal side effects.

- Many "addicts" of breath-freshening products are ashamed and embarrassed regarding their own perceived malodor. Such individuals are willing to buy new products anonymously because they are often dissatisfied with current ones. But, because of their embarassment, they are likely to shy away from participating in "shopping mall" consumer testing and thus may be excluded from market analyses.
- Dentists are expected to play an increasingly important role in marketing breath-freshening mouthrinses and other products directly to the consumer via "fresh breath clinics." Since a dentist's recommendation of a given product can convince even a skeptical patient, I expect that this already considerable niche will continue to grow in coming years.

Mel Rosenberg invites interested readers to contact him directly through his e-mail address: melros@post.tau.ac.il. Also, Tel Aviv University invites scientists interested in oral malodor research to visit its homepage on bad-breath research for information on related articles and seminars: http://www.tau.ac.il/~melros/mel1.html.

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For More Information

Assuta ..... Circle No. 151
Portable sulfide monitor ..... Circle No. 152

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Dear Dr. Rosenberg:

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Chapter 17

29

HYDROPHOBIC INTERACTIONS AS A BASIS FOR INTERFERING WITH MICROBIAL ADHESION

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INTRODUCTION

Hydrophobic interactions are considered important in a variety of microbial adhesion phenomena in both the open environment, as well as the host (for a review on hydrophobicity in the context of microbial adhesion, see Rosenberg and Doyle, 1990). Examples of medically-related adhesion phenomena that appear to be influenced by hydrophobic interactions include adhesion to teeth, fatty tissues, insect cuticle, catheters, intrauterine contraceptive devices, bioprostheses, contact lenses, and other biomaterials, as well as a variety of epithelial and phagocytic cell types. In 1980, we proposed a simple technique for measuring bacterial (later including eucaryotic microorganisms) adhesion to liquid hydrocarbons based on simply vortexing washed cell suspensions with different test hydrocarbons, with concomitant adhesion at the oil:water interface. The observation that adhesion could be reversed by isopropanol was one indication that hydrophobic interactions were involved, leading us to propose this test as a simple technique for studying cell surface hydrophobicity (Rosenberg *et al.*, 1980). Many tests exist for studying hydrophobic surface properties of microorganisms, including some recently proposed techniques (Lin *et al.*, 1995), but their discussion is beyond the scope of this chapter (for reviews, see Rosenberg and Doyle, 1990; Rosenberg, 1991; Rosenberg *et al.*, 1991a; Van der Mei *et al.*, 1991). The microbial adhesion to hydrocarbon (MATH) test subsequently led us into various avenues of investigation related to identification of cell surface components which promote or interfere with adhesion at the oil:water interface, as well as soluble agents which inhibit or promote adhesion. Furthermore, the observation that many oral microorganisms adhere at the oil:water interface led us to develop a novel two-phase microbe-desorbing mouthrinse, which has been sold successfully in Israel since 1992.

Toward Anti-Adhesion Therapy for Microbial Diseases, edited by Kahane and Ofek
Plenum Press, New York, 1996

HYDROPHOBINS AND HYDROPHILINS

In 1986, Rosenberg and Kjelleberg coined the term "hydrophobin" to denote hydrophobic surface components that promote cell surface hydrophobicity and adhesion. Conversely, hydrophilins were defined as cell surface components that reduced hydrophobic surface properties of microbial cells. In recent years the term hydrophobin has been extended to include hydrophobic surface components of fungal cells that may be involved in adhesion processes. Studies on hydrophobins and hydrophilins of several microbial species are discussed below.

ACINETOBACTER CALCOACETICUS

Acinetobacter calcoaceticus species, although often found in the environment, can inhabit the human body and are sometimes associated with nosocomial infections. Stationary phase cells of the oil-degrading *A. calcoaceticus* RAG-1 exhibit a high affinity for the oil:water interface. Mutant MR-481, selected for its inability to adhere to oil droplets, was devoid of thin fimbriae present on wild type cells. Revertants bearing thin fimbriae regained partial adhesion characteristics. These data established a role for the thin fimbriae as a hydrophobin of RAG-1 cells (Rosenberg *et al.*, 1982). However, another RAG-1 mutant was found to be able to adhere at the oil:water interface, although it lacked thin fimbriae. Thus, other RAG-1 hydrophobin(s) remain to be elucidated.

Emulsan, a capsular anionic heteropolysaccharide of *A. calcoaceticus* RAG-1 acts as a hydrophilin when present on RAG-1 cells. When sloughed off in solution, emulsan is a potent inhibitor of microbial adhesion (see further). Similarly, the rhamnose-rich capsule of *Acinetobacter calcoaceticus* BD 4, although completely different in composition, also acts as a hydrophilin. As the capsule is enzymatically degraded, adhesion to oil droplets rises from zero to almost 100% (Rosenberg *et al.*, 1983b).

STREPTOCOCCUS PYOGENES

Streptococcus pyogenes is a common cause of throat infections, and other, sometimes serious, diseases. Cells of *S. pyogenes* often adhere avidly to hexadecane and other oil droplets (Ofek *et al.*, 1983). LTA and M protein have both been considered as contributing to the hydrophobic surface properties. Conversely, hyaluronate capsule appears to act as a hydrophilin, and the hydrophobic/hydrophilic conversion of cells may be critical for successful colonization.

SERRATIA MARCESCENS

Serratia marcescens is a ubiquitous microbial species which can be a troublesome source of nosocomial infections, particularly in neonate wards. It has been known for some time that prodigiosin, the red, oil-soluble pigment characteristic of pigmented strains imparts hydrophobic surface characteristics, enabling cells to partition at air:water interfaces. However, most clinical isolates are nonpigmented. Nevertheless, nonpigmented strains are also capable of adhering (albeit, less avidly) to hydrocarbons and polystyrene (Rosenberg *et al.*, 1986). Furthermore, growth of cells at 37°C leads to loss of pigment, whereas cells are still adherent. Finally, we have isolated pigmented mutants which lost their surface hydropho-

bicity as well as nonpigmented mutants that retained partial hydrophobic surface characteristics (Rosenberg *et al.*, 1984a). In 1989, Bar Ness and Rosenberg showed that a 70 kDa *S. marcescens* (wild type) surface protein, serraphobin, binds at the oil:water interface and is associated with cell surface hydrophobicity (Bar Ness-Greenstein, 1992). Transformation of *Escherichia coli* with DNA from *S. marcescens* resulted in a transformant clone exhibiting an increase in hydrophobic surface properties, concomitant with appearance of *S. marcescens* antigen/s (as yet unidentified) on the surface of transformant cells. Rabbit antiserum raised against whole cells of wild type hydrophobic strain of *S. marcescens* (and adsorbed on the appropriate control cells), reacted with the transformant in colony immuno-blotting. The insert size of the hydophobic transformant was 5.4 kb, as revealed by restriction mapping (BarNess-Greenstein *et al.*, 1995).

Another amphipathic cell surface component, the cyclic aminolipid, serratamolide, turned out to act as a hydrophilin. Mutants lacking this surfactant molecule exhibited higher adhesion levels than isogenic wild type cells (Bar-Ness *et al.*, 1988).

CANDIDA ALBICANS

Candida albicans is an opportunistic yeast-like microorganism which may give rise to superficial and systemic infections. Although hydrophobic surface properties of *C. albicans* were found in several investigations (Calderone & Braun, 1991; Hazen *et al.*, 1991; Tronchin *et al.*, 1988) the cell surface components involved are unclear. In order to look for surface hydrophobin(s) of this microorganism, a genomic library of *C. albicans* in a nonadherent strain of *Saccharomyces cerevisiae* was constructed. Selection for adhesion to polystyrene yielded a transformant which exhibited enhanced adhesion to treated and untreated polystyrene, as well as pronounced autoaggregation, as compared to appropriate control cells bearing the vector alone. Analysis of this clone revealed an insert of ca. 4.5 kb from *C. albicans*. Curing of the plasmid resulted in loss of adhesion and autoaggregation properties (Barki *et al.*, 1993). A subclone bearing a reduced insert of 3.3 kb retained the ability to autoaggregate, to bind to treated and untreated polystyrene, and to adhere to buccal epithelial cells, as compared to appropriate controls. Evidence was subsequently presented that the adhesion/autoaggregation phenotype observed in *S. cerevisiae* cells transformed with the candidal DNA fragment is due to expression of a *C. albicans* surface antigen (Barki *et al.*, 1994). Rabbit antiserum, raised against transformant *S. cerevisiae* cells, was adsorbed on *S. cerevisiae* bearing the vector alone. Immunofluorescence micrography showed that the adsorbed antisera bound to the surface of transformant *S. cerevisiae* cells as well as to *C. albicans* cells (especially to the invasion-associated mycelial phase), but only marginally to the *S. cerevisiae* control. Furthermore, adsorbed antisera specifically inhibited autoaggregation of transformant cells. Autoaggregative activity was also abolished following treatment by trypsin and proteinase K. Western immunoblot analysis yielded an antigen of ca. 30 kDa molecular weight, present in transformant cell surface extracts, but absent in the control. The extent to which this antigen acts as an adhesin/hydrophobin on *C. albicans* cells is currently under investigation.

SOLUBLE AGENTS THAT AFFECT MICROBIAL ADHESION VIA PROMOTION OF OR INTERFERENCE WITH HYDROPHOBIC INTERACTIONS

A wide variety of amphipathic substances may inhibit adhesion to animate and inanimate surfaces (for a review see Rosenberg and Doyle 1990). Some examples are

detergents (e.g., sodium dodecyl sulfate, Tween 80, Tween 20, Triton X-100), chaotropes (e.g., thiocyanate anion), components obtained from body fluids (e.g., bovine serum albumin [particularly defatted], normal human serum, salivary components, caseinoglycopeptides) and small organic molecules (e.g., p-nitrophenol, tetramethyl urea, propanol, fatty acids).

Amphipathic components in saliva appears to act as a barrier, preventing free microorganisms from adhering to oral surfaces. Washed microorganisms obtained directly from dental plaque were capable of adhering to buccal epithelial cells in vitro, but this was largely inhibited when saliva was added (Rosenberg *et al.*, 1989). Saliva prevented microbial adhesion to polystyrene, but when the saliva was extracted with hexadecane, it lost its inhibitory ability (Eli *et al.*, 1989). Babu and coworkers demonstrated in 1986, inhibition of adhesion of *Streptococcus sanguis* to hexadecane by specific hydrophobic proteins of saliva.

Adhesion of microorganisms to hexadecane and polystyrene is promoted in the presence of ammonium sulphate (Rosenberg *et al.*, 1984b). As discussed further below, amphipathic cationic molecules (e.g., cetylpyridinium chloride, chlorhexidine, lysozyme, poly L-lysine, chitosan) may promote adhesion by "hydrophobizing" cells. This presumably occurs by binding of such molecules to negatively charged cell surface residues, thereby simultaneously decreasing surface charge and adding hydrophobic moieties to the cell outer surface (Goldberg *et al.*, 1990a and 1990b). Covalent blocking of negatively-charged outer surface components similarly results in increased hydrophobicity and adhesion.

One interesting aspect is the inhibition by amphipathic molecules of adhesion apparently mediated by stereospecific, lectin-like interactions. Adhesion of *E. coli* to epithelial cells, while sensitive to mannose, can also be blocked by unrelated amphipathic compounds, such as p-nitrophenol (Falkowski *et al.*, 1986). *Actinomyces viscosus* cells preferentially adhered to *Porphyromonas gingivalis* cells, rather than to hexadecane, yet this ostensibly specific interaction was inhibited by defatted bovine serum albumin (Rosenberg *et al.*, 1991b).

The therapeutic use of anti-adhesion agents in controlling microbial infections must consider that in most cases, we become aware of the intruding microorganisms after they have already adhered. In other words, for most medical purposes, anti-adhesion agents must be able to desorb already attached microorganisms, as well as to inhibit adhesion. Unfortunately, prevention of initial attachment is easy, as compared with detaching microorganisms after they have already adhered, interacted with neighbouring bacteria, elaborated a capsular glycocalyx, and so on.

Relatively few studies have dealt with microbial desorption. In one such investigation, E. Rosenberg and coworkers were able to show that when the capsular anionic heteropolysaccharide of *A. calcoaceticus* RAG-1 ("emulsan") is sloughed off into the aqueous phase, it is not only capable of inhibiting adhesion to buccal epithelial cells, but also desorbs a substantial fraction of the indigenous attached microbiota (Rosenberg *et al.*, 1983a).

Another consideration facing anti-adhesion therapy is the observation that bacteria will eventually adhere to and foul practically every available interface. As Kevin C. Marshall has succinctly remarked in this context, "The Bacteria Always Win." In the following section, we describe an alternative anti-adhesion strategy, i.e., the BAWA ("beat adhesion with adhesion") approach. According to this strategy, adhering bacteria can be desorbed from one interface by preferential adhesion to a second interface (Rosenberg *et al.*, 1983c). We have successfully used oil droplets to remove bound bacteria from oral surfaces in this manner (Goldberg and Rosenberg, 1991). It is important to emphasize that in such an instance, the mechanisms governing the initial adhesion are probably of secondary importance, as compared with the affinity of the bound cells for the displacing interface.

FROM OIL POLLUTION TO ORAL POLLUTION: RESEARCH AND DEVELOPMENT OF A BACTERIA-DESORBING MOUTHWASH

Over the past thirteen years, much of our interest in anti-adhesion therapy has focused on the potential of two-phase, oil:water mixtures to remove oral bacteria and to combat bad breath. In 1982, Nesbitt and coworkers showed that *Streptococcus sanguis* adheres to toluene droplets. In the same year, Weiss *et al.* reported that most oral microbial isolates retrieved from extracted teeth adhered to hexadecane. In 1983, Rosenberg and coworkers subsequently demonstrated that washed, dispersed suspensions of microorganisms obtained directly from supragingival dental plaque adhered in high proportions to droplets of liquid hydrocarbon (Rosenberg *et al.,* 1983d). These initial observations led to the notion of using two-phase oil:water mouthwashes for physical desorption of oral microbiota. Indeed, other studies have demonstrated that a wide variety of laboratory strains of oral microorganisms, including *Actinobacillus actinomycetemcomitans, Actinomyces viscosus and Actinomyces naeslundii, Porphyromonas gingivalis, Streptococcus salivarius, Streptococcus sanguis*, and certain *Streptococcus mutans* strains, adhered with high affinity to hydrocarbons (for a review, see Doyle *et al.,* 1990). Initial experiments showed that microorganisms which adhere to hydrocarbons also bind to other nontoxic oils (Rosenberg *et al.,* 1983c). Furthermore, in vitro experiments showed that water:oil combinations could desorb bacteria from plastic surfaces. Desorption could also be demonstrated *in vivo*, based on removal of erythrosine-stained bacteria and debris from the mouth following rinsing with a two phase rinse consisting of saline and olive oil (Weiss *et al.*, 1985). Similar combinations were also able to remove bound cells from polystyrene cuvettes (Goldberg and Rosenberg, 1991).

Despite these initial desorption experiments, the simple aqueous:oil combinations did not prove particularly effective in improving oral hygiene over time. Furthermore, the two immiscible oil and water phases posed technical difficulties. In 1989, Goldberg and coworkers found that adhesion of microorganisms and oral debris to oil was promoted by low concentrations of cetylpyridinium chloride (CPC), an amphipathic cationic agent which is commonly added to mouthwashes. This was surprising, since amphipathic agents were generally considered to inhibit adhesion at the oil:water interface. Since CPC is an antibacterial agent, its addition had several other advantages, including inhibition of microbial activity in the mouth, and increasing shelf life of the product by preventing microbial outgrowth. Furthermore, addition of an amphipathic agent such as CPC to oil:water mixtures results in a temporary emulsion following shaking. This in turn enabled the mixture to be supplied in a single bottle.

Prototype formulations, consisting of an aqueous phase containing CPC, and an oil phase comprising a mixture of vegetable and essential oils, were subsequently developed and tested. Such formulations maintained dramatic *in vitro* bacterial desorption properties, removing over 90% of a bacterial film of *A. calcoaceticus* RAG-1 on polystyrene cuvettes, as compared to 15-50% removal for a series of commercial mouthwashes (Goldberg and Rosenberg, 1991).

In an initial clinical study, the prototype two-phase rinse was compared with (i) a placebo rinse; and (ii) a mouthrinse containing 0.2% chlorhexidine gluconate (Corsodyl™, ICI). Subjects performed oral rinsing prior to bedtime and in the morning of the following day. Measurements were taken in the late afternoon (e.g. at least eight hours following rinsing) of the second day, and were compared to those taken the previous afternoon prior to rinsing. The data demonstrated that rinsing with the two-phase mouthwash resulted in daylong reduction in microbial levels, as well as in oral malodor (Rosenberg *et al.,* 1992). In another study employing the same two phase formulation, Yaegaki and Sanada showed dramatic reductions in volatile sulphide levels 3.5 hours after use, as compared to a

commercial mouthwash (Skoal™). These authors suggested that physical desorption of the particulate oral debris by the mouthrinse was an important factor in malodor reduction (Yaegaki and Sanada, 1992).

In 1992, the prototype two-phase oil-water mouthwash was reformulated and introduced in the Israeli market. A clinical study (Kozlovsky *et al.*, in press) was performed to compare the efficacy of the commercial version of the two-phase mouthwash with Listerine™, a mouthrinse which has been previously shown to be effective in reducing levels of odor-related microorganisms, as well as plaque and gingivitis (Pitts *et al.*, 1983; DePaola *et al.*, 1989). In the study, fifty subjects rinsed with one of the two mouthwashes for 30 seconds twice a day over six weeks, while continuing their regular oral hygiene habits. In both mouthwash groups, dramatic improvements were observed in parameters associated with oral microbial levels, malodor, periodontal health and plaque accumulation. For example, as compared to time zero scores, whole mouth odor as well as tongue dorsum anterior and posterior odors decreased continuously over time, attaining 80%, 79% and 70% reductions, respectively, following 6 weeks, in the two-phase mouthwash (Assuta™) group, vs. 70%, 77% and 59% for the Listerine™ group. For whole mouth and tongue dorsum posterior, the reductions observed in the two-phase mouthwash group were significantly greater than those obtained with Listerine™ ($p=0.026$ and $p=0.025$, respectively), suggesting that the two-phase mouthwash is superior to Listerine™ in long-term reduction of oral malodor.

CONCLUDING REMARKS

Hydrophobic interactions appear to be involved in numerous examples of microbial adhesion. Whereas many amphipathic agents (detergents, chaotropes, etc.) can inhibit adhesion which is mediated via hydrophobic interactions, desorption of already-bound cells (e.g., from dental plaque) is a more difficult prospect. Emulsan, a capsular polysaccharide from *A. calcoaceticus* RAG-1 is one example of a bacteria-desorbing polymer. Unfortunately, this potential, demonstrated many years ago, has not been further exploited. One approach which works for the oral cavity is the "beat adhesion with adhesion" approach, in which attached bacteria are simultaneously hydrophobized (by cetylpyridinium chloride) and desorbed onto oil droplets. Further research is necessary to determine whether similar techniques can be used to desorb bacterial films from other surfaces of medical interest.

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Efficacy of a 2-Phase Oil:Water Mouthrinse in Controlling Oral Malodor, Gingivitis, and Plaque

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THE PURPOSE OF THE STUDY WAS TO EXAMINE THE ANTI-MALODOR, anti-gingivitis, and plaque reducing properties of a 2 phase oil:water mouthrinse compared with a control mouthrinse. Fifty subjects rinsed with one of the two rinses for 30 seconds twice a day over 6 weeks, while continuing their normal oral hygiene habits. Measurements were made at time zero (prior to beginning the rinsing regimen), and ≥ 9 hours following rinsing, at intervals of 1, 3, and 6 weeks. Malodor of whole mouth, as well as tongue dorsum anterior and posterior, was assessed on a 0 to 5 semi-integer scale by two odor judges. Volatile sulphide compounds (VSC) were determined using a sulphide monitor. Gingival, plaque, and bleeding indices were recorded for Ramfjord teeth. Oral microbial levels were assessed using the oratest. Salivary levels of diamines (putrescine and cadaverine) were analyzed by HPLC. Results were analyzed by 2-tailed covariant ANOVA, with the time zero value as covariant. Dramatic improvements were observed in parameters associated with malodor, periodontal health, plaque accumulation, and microbial levels in both groups. As compared to time zero scores, whole mouth odor, tongue dorsum anterior and posterior odors decreased continuously over time, attaining 80%, 79% and 70%, reductions, respectively following 6 weeks, in the 2-phase mouthrinse group, versus 70%, 77% and 59% for the control group. For whole mouth and tongue dorsum posterior, the reductions observed in the 2-phase mouthrinse group were significantly greater than those obtained with the control mouthrinse ($P = 0.026$ and $P = 0.025$, respectively), suggesting that the 2-phase mouthrinse is superior to the control mouthrinse in long-term reduction of oral malodor. For bleeding index, gingival index, oral microbial levels, and VSC, differences between the groups were not significant. Diamine levels were not significantly reduced in either group. The control mouthrinse reduced plaque index more significantly than the 2-phase mouthrinse ($P < 0.005$). The results of this randomized clinical trial suggest that the 2-phase oil:water mouthrinse formulation is superior to the control mouthrinse in long-term reduction of oral malodor. *J Periodontol 1996;67:577–582.*

Key Words: Halitosis/prevention and control; gingivitis/prevention and control; dental plaque/prevention and control; mouthrinses/therapeutic use; comparative study.

Mouthrinsing is a common oral hygiene practice dating back to ancient times.[1] The major concern which leads to frequent use of mouthrinses is halitosis (bad breath).[2] Bad breath usually originates within the oral cavity itself, due to production of gases (primarily volatile sulphide compounds, VSC) by sequestered deposits of microorganisms, generally under anaerobic conditions.[3–5] Periodontal diseases appear, in most studies, to be related to bad breath.[4–6] Other loci of oral microbial putrefaction include the tongue dorsum, areas of food impaction, and improper dental restorations.[4] Secondary reasons for use of a mouthrinse include control of plaque and gingivitis when used as an adjunct to mechanical means.[1–3]

Much of the evidence of the efficacy of mouthrinses in reducing bad breath is anecdotal,[7] and there are very few publications in the scientific literature on this subject. Long-term malodor reduction has rarely, if ever, been addressed. For example, in investigations carried out by

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Pitts et al.,[8] Schmidt and Tarbet,[9] and Tonzetich,[10] data on malodor reduction were collected only up to 3 hours following use.

Research has shown that 2-phase, oil:water formulations containing cationic surface-active agents such as cetylpyridinium chloride (CPC), efficiently bind[11] and desorb[12,13] oral microorganisms. Two studies have been published on the anti-malodor effects of a prototype 2-phase mouthwash formulation of this type. In the first study, Rosenberg et al.[14] compared the 2-phase prototype rinse with a placebo rinse and an antiseptic mouthrinse containing 0.2% chlorhexidine gluconate. Subjects performed oral rinsing prior to bedtime and in the morning of the following day. Measurements were taken in the late afternoon (e.g., at least 8 hours following rinsing) of the second day and were compared to those taken the previous day afternoon prior to rinsing. The data demonstrated that mouthrinsing with the 2-phase mouthwash results in daylong reduction in bad breath-associated parameters.[14] In another study, Yaegaki and Sanada[15] showed dramatic reductions in volatile sulphide levels 3.5 hours after a single use of the same prototype mouthrinse, as compared to time zero measurements, as well as in comparison to a commercial mouthwash.

Since these experiments were performed, a reformulated 2-phase oil:water mouthrinse[§] containing CPC was introduced in the Israeli market. The purpose of the present study was to test the efficacy of this mouthrinse in controlling oral malodor, plaque, and gingivitis over a 6-week period, as compared with a mouthrinse[‖] which has been previously shown to be effective in reducing levels of odor-related microorganisms, as well as plaque and gingivitis.[8,16,17]

MATERIALS AND METHODS

The experiment consisted of a 6-week, randomized clinical trial of 50 volunteers (mean age 24 years; 37 females). Volunteers were recruited by advertisements placed on the campus of Tel Aviv University and were remunerated. Smokers and partial denture wearers were excluded. Following randomized distribution into one of the two mouthrinse groups, subjects received either the 2-phase mouthwash (N = 26) or the control mouthrinse (N = 24). Volunteers rinsed morning (following breakfast and toothbrushing) and evening (directly prior to bedtime, following toothbrushing). Each rinse consisted of 30 seconds vigorous mixing in the mouth and gargling. For the 2-phase mouthwash, volunteers were instructed to shake the bottle before use. Volunteers were asked to refrain from rinsing with water, eating, or drinking, for at least 30 minutes following rinsing. During the course of the 6-week trial, the participants continued their usual oral hygiene and dietary habits but were instructed to refrain from using other commercial mouthrinses. Measurement days were day zero (baseline prior to rinsing), and 1, 3, and 6 weeks. Volunteers were not subjected to any prophylactic treatment. On measurement days, volunteers were instructed not to apply scented products; not use the mouthrinse before arrival; and to refrain from eating, drinking, or chewing gum for at least 2 hours prior to examination.

Measurement Parameters

Soft tissue examination. A complete intraoral soft tissue examination was carried out at each appointment to evaluate the condition of the oral mucosa. The buccal, labial, and sublingual mucosa; the tongue; the hard and soft palate; the uvula; and the oropharynx were examined for inflammation, ulcerations, or other lesions. Aberrations were recorded, their severity assessed, and a judgement made as to whether they were attributable to the mouthrinse preparations and regimens utilized.

Gingival index. Modified gingival index was determined according to Lobene et al.,[18] employing Ramfjord teeth.[19] Results were scored as mean of all surfaces examined.

Plaque index. Plaque area was scored by the Turesky modification of the Quigley-Hein index,[20] employing Ramfjord teeth. Results were scored as mean of all surfaces examined.

Bleeding index. Papillary bleeding index was determined according to Muhlemann,[21] employing Ramfjord teeth. Results were scored as mean of all surfaces examined.

Volatile sulfur compounds (VSC). Quantitative measurement of intraoral volatile sulphides (VSC) was carried out using a sulphide monitor,[¶] 1 ppm full-scale, connected to a pen recorder. Measurement was essentially as previously reported,[22,23] except that disposable straws, rather than teflon tubing, were employed. Volunteers were asked to refrain from talking for several minutes prior to measurement. The monitor was zeroed on ambient air, and measurement performed by inserting a disposable ¼" plastic straw approximately 4 cm into the partially opened oral cavity. The volunteer was instructed to breathe through his/her nose during measurement. Results were recorded as peak ppb sulphide equivalents.

Oral microbial levels. Oral microbial levels were determined using the oratest, a technique which measures the rate of oxygen depletion in expectorated milk samples. The technique has been previously described and shown to correlate with microbial counts and clinical parameters.[14,24–26] Briefly, volunteers rinsed for 30 seconds with 10 mL of sterile milk. Following expectoration, 3 mL of the samples were added to test tubes containing

[§]Assuta, Shemen Soad Industries, Ltd., Haifa, Israel.
[‖]Listerine, Warner-Lambert, Morris Plains, NJ.
[¶]Model 1170, Interscan Corp., Chatsworth, CA.

Table 1. Reductions in Malodor Related Parameters over Time*

Parameter	Baseline	Week 1	Week 3	Week 6
Whole mouth (scale of 0–5)				
Experimental†	2.14 ± 0.88	0.85 ± 0.83	0.69 ± 0.69	0.42 ± 0.55
Control	2.40 ± 1.00	1.38 ± 0.84	1.29 ± 0.78	0.71 ± 0.64
Tongue dorsum anterior (scale of 0–5)				
Experimental‡	1.43 ± 0.95	not determined	0.59 ± 0.39	0.30 ± 0.26
Control	1.91 ± 0.98	not determined	0.95 ± 0.66	0.43 ± 0.45
Tongue dorsum posterior (scale of 0–5)				
Experimental	2.50 ± 0.79	not determined	1.34 ± 0.86	0.75 ± 0.60
Control	2.87 ± 1.04	not determined	1.95 ± 0.76	1.18 ± 0.82
Peak volatile sulphide (ppb hydrogen sulfide equivalents)				
Experimental	94 ± 36	58 ± 14	52 ± 11	56 ± 10
Control	79 ± 40	69 ± 33	58 ± 16	56 ± 16

*Results are expressed as mean ± standard deviation.
†Significantly lower over time as compared with control ($P = 0.026$).
‡ Significantly lower over time as compared with control ($P = 0.025$).

0.12 mL of methylene blue solution (0.1%), and the time required for a blue-to-white color change over a 6 mm diameter at the bottom of the test tube recorded. The test was performed directly following the VSC measurements.

Odor judge measurements. Organoleptic measurements were made by two odor judges, based on the whole mouth expirate, as well as odor assessment from the anterior and posterior of the tongue dorsum.[27,28] For whole mouth malodor, following a 2-hour fast, subjects were instructed to exhale briefly through the mouth, at a distance of about 10 cm from the nose of the judge. Odor from the anterior tongue dorsum was determined by having the volunteers lick their wrist with their extended tongue. After 5 seconds, the odor judge smelled the odor at a distance of about 5 cm from the wrist of the volunteer. For assessment of the posterior tongue dorsum, samples were obtained by mild scraping of the area with a plastic spoon. Following 5 seconds, the odor judge scored the odor at a distance of about 5 cm from the spoon. Results of each malodor assessments were rated on a semi-integer scale from 0 to 5 with descriptions as follows: 0: no appreciable odor; 1: barely noticeable odor; 2: slight, but clearly noticeable odor; 3; moderate odor; 4: strong odor; 5: extremely foul odor. The average of the two judges scores was used for each measurement. Both judges were blinded to one another's scores, as well as to the mouthrinse used by each volunteer.

Diamine analysis. Unstimulated saliva samples (200 μL) were passed through 0.45 μm pore filters and were then analyzed essentially as described by Goldberg et al.[29] Samples from 8 subjects could not be filtered and were not included in the analysis. For high performance liquid chromatography (HPLC) analysis, samples were first derivitized using O-phthaldialdehyde which was prepared by dissolving 50 mg of O-phthaldialdehyde in 4.5 mL methanol, together with 0.5 mL borate buffer (0.9 M, pH = 9.5) and 50 μL of β-mercaptoethanol. The solution was diluted 1:10 in methanol and kept at −20°C. Eighty μL borate buffer and 25 μL of the diluted reagent solution were added to saliva samples (20 μL). The HPLC system consisted of pump,** an injection valve equipped with a 20 μL sample loop, and a C18 reverse phase column†† (250 × 4 mm). Samples were injected at a flow rate of 1 mL/min. The mobile phase was composed of a filtered, degassed 30:70 mixture of 12.5 mM sodium phosphate buffer (pH = 7.2):acetonitrile. Detection was carried out using a UV detector‡‡ at a wavelength of 231 nm.

Statistical analysis. To approximate normal distributions, bleeding index levels, oral microbial level scores and salivary concentrations of putrescine and cadaverine were ln transformed. Statistical comparisons of these parameters were thus performed on ln transformed values.[14,24–26] Since, in several instances, significant differences were found between the two groups at baseline when compared using the unpaired T test, the data were subsequently analyzed by 2-tailed ANCOVA, with repeated measures, using the time zero value as covariant. Pearson correlation coefficients were employed to test for correlations among the results of the different measurement techniques on all 50 participants at baseline and following 3 weeks.

RESULTS

Results of the oral malodor-related scores (average of judge scores of whole mouth, tongue dorsum anterior, and tongue dorsum posterior, as well as VSC), are summarized in Table 1. Results of the oral indices (gingival index, bleeding index, plaque index) and oral microbial lev-

**Eldex 9600, Eldex Laboratories, Inc. Napa, CA.
§§Lichrosphere 100 PR-18, Merck, Darmstadt, Germany.
‡‡Spectro Monitor 3,200 LDC Analytical, Orlando, FL.

Table 2. Dental and Microbiological Parameters Over Time*

Parameter	Baseline	Week 1	Week 3	Week 6
Gingival index				
Experimental	1.30 ± 0.58	1.19 ± 0.52	0.89 ± 0.51	0.62 ± 0.44
Control	1.58 ± 0.52	1.44 ± 0.51	1.14 ± 0.43	0.80 ± 0.44
Bleeding index				
Experimental	0.33 ± 0.28	0.35 ± 0.30	0.24 ± 0.25	0.22 ± 0.26
Control	0.48 ± 0.26	0.50 ± 0.33	0.39 ± 0.23	0.30 ± 0.27
Plaque index				
Experimental	1.86 ± 0.61	1.41 ± 0.64	1.13 ± 0.52	0.95 ± 0.50
Control†	1.92 ± 0.65	1.03 ± 0.68	0.86 ± 0.66	0.72 ± 0.61
Oratest level (minutes for color change)				
Experimental	124 ± 305	214 ± 181	395 ± 275	672 ± 394
Control	101 ± 105	267 ± 171	480 ± 435	617 ± 310

*Results are expressed as mean ± standard deviation.
†Significantly lower over time as compared with experimental (P = 0.005).

Table 3. Changes in Diamine Concentration*

Parameter	Baseline	3 Weeks
Putrescine		
Experimental	9.6 ± 7.0	10.4 ± 10.0
Control	11.5 ± 11.5	12.0 ± 8.9
Cadaverine		
Experimental	4.7 ± 7.4	4.5 ± 3.6
Control	4.8 ± 5.3	4.0 ± 3.0

*Mean ± standard deviation (ppm).

el scores are presented in Table 2. Cadaverine and putrescine measurements are summarized in Table 3.

In general, highly significant improvements in parameters associated with malodor, periodontal health, and plaque accumulation were observed in both groups. In oral malodor-related parameters, greater reductions were observed following use of the 2-phase mouthrinse, as compared to the control. Following 6 weeks use of the 2-phase mouthrinse, mean whole mouth odor and tongue dorsum anterior and posterior odors were reduced by 80%, 79%, and 70%, respectively, as compared to corresponding mean reductions of 70%, 77%, and 59% in the control group. In the case of whole mouth and tongue dorsum posterior, the reductions observed in the 2-phase group were significantly greater than those obtained with the control (P = 0.026 and 0.025, respectively). In both groups, the reductions over time were highly significant for all three odor judge scores ($P < 0.0001$). Following 6 weeks use, mean volatile sulphides were reduced by 40% in the 2-phase mouthrinse groups, as compared with 29% in the control group, but this difference was not significant (P = 0.16).

In the case of periodontal-related parameters; i.e., gingival index and bleeding index, highly significant reductions compared to baseline ($P < 0.0001$) were observed in both groups over time. Following 6 weeks of use, mean gingival index scores were similarly reduced in the 2-phase mouthrinse and control groups by 52% and 49%, respectively. Comparable reductions in mean bleeding index scores were also observed following 2 weeks (33% and 37% for the 2-phase mouthrinse and control groups, respectively). In the case of plaque index, highly significant reductions over time were observed in both groups, as compared to baseline ($P < 0.0001$). Whereas mean reduction in both bleeding and gingival indices were comparable in both experimental and control groups, the mean reduction in plaque index in the control group (62%) was significantly lower than the reduction observed in the 2-phase group (49%; P = 0.005). Highly significant increases ($P < 0.0001$) were observed in oratest level scores in both groups, indicative of reductions in microbial levels.[24–26] Differences in experimental versus control scores were not significant over time.

The salivary levels of cadaverine and putrescine at baseline and following 3 weeks are compared in Table 3. In contrast to all other parameters, significant reductions were not observed in either group.

Pearson correlation coefficients were computed to compare associations among the various parameters. At baseline, significant correlations were observed between oral microbial level scores and both whole mouth odor (P = 0.021), as well as tongue posterior odor (P = 0.010); a marginal association was observed between oral microbial level results and bleeding index scores (P = 0.062). Putrescine levels were inversely related to tongue anterior odor scores (P = 0.024). As expected, diamine scores were significantly associated with one another (P = 0.005). Similarly, whole mouth, and tongue anterior and tongue posterior scores were all closely related to one another ($P < 0.01$). Gingival index was closely related to bleeding index ($P < 0.001$), and was associated with plaque index (P = 0.010) and tongue anterior odor (P = 0.038). Correlations were also sought in comparing the differences between baseline and 3 weeks scores for all subjects. Changes in oratest level scores were associated with changes in both tongue posterior odor (P = 0.012) and cadaverine (P = 0.020). Changes in putrescine were also associated with changes in posterior tongue odor (P = 0.27), as well as with changes in bleeding index scores (P = 0.001). The changes in levels of the two diamines following 3 weeks were highly correlated with each other ($P < 0.001$).

DISCUSSION

The main purpose of the present study was to test the efficacy of a novel 2-phase mouthrinse on oral malodor and dental-associated parameters. A mouthrinse with previously demonstrated effectiveness in reducing malodor, gingivitis, bleeding, and plaque and oral microbial levels[8,16,17,30] was employed as a control. Subjects were measured at baseline, and were then instructed to use one of

the rinses twice a day over a 6-week period, as a supplement to their usual oral hygiene habits. Measurements were conducted at 1, 3, and 6 weeks.

The data show that the 2-phase mouthwash is highly effective in reducing bad breath parameters. Significantly greater reductions in whole mouth and tongue posterior malodor were observed in the 2-phase mouthwash group, as compared with the control mouthrinse. Considering the fact that measurements were performed 9 to 18 hours following the previous rinsing, the mean reductions observed (e.g., 80% in the 2-phase mouthwash group, following 6 weeks) are particularly noteworthy.

Highly significant and comparable reductions in gingivitis levels, as measured by gingival index, were observed in both mouthrinse groups. The mean reductions observed in the experiment and control groups (52% and 49% respectively) are comparable to the results found in a 6-week study conducted by Axelsson and Lindhe.[31] Furthermore, reduction in bleeding and microbial load were highly significant and similar in both groups.

Although both mouthrinses significantly reduced plaque levels in comparison to baseline, the control significantly outperformed the two-phase mouthrinse, with a 63% mean reduction following 6 weeks, as compared to 49%. In the Axelsson and Lindhe study,[31] mean plaque reduction by the control was 51%, and was found to be comparable to that achieved by 0.1% chlorhexidine (54%). The role of supragingival plaque in malodor is at present unclear.[6] In several investigations, plaque index was significantly associated with oral malodor.[22,27] However, in a more recent study, a significant correlation was not observed.[32] Similarly, in the present study, baseline plaque levels were not associated with any of the odor-related parameters measured ($P > 0.05$). Since the 2-phase mouthrinse appeared more effective than the control in reducing malodor (especially with respect to the tongue dorsum posterior), but was relatively less effective in supragingival plaque reduction, we may postulate that the superior malodor-reducing effect of the 2-phase mouthrinse is related primarily to its efficacy in reducing the load of odorigenic microorganisms on the tongue dorsum posterior.

Previous investigations have suggested that the potent anti-malodor and antibacterial properties of 2-phase oil: water formulations stem mainly from the desorption of oral bacteria and debris by oil droplets.[12,15] Investigations are currently underway to determine whether additional mechanisms, such as slow release of CPC, potentiated by the oil droplets, may also play a role in the efficacy of such compositions.

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The Complaint of Oral Malodor: Possible Psychopathological Aspects

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Oral malodor (halitosis) is a common concern in Western society. As with other human perceptions, emotional as well as cognitive variables play a major role in one's sensation and complaint. To study factors potentially associated with the complaint of oral malodor, periodontal and psychological evaluations were carried out on 38 subjects (66% female, mean age 43 years) with a complaint of oral malodor. Subjects underwent evaluation of their periodontal status, odor evaluation by an odor judge, and psychopathological symptom survey by means of the SCL-90 questionnaire. The patient's self-rating of oral odor was significantly higher than the evaluation of an objective odor judge and was not associated with their periodontal status. The SCL-90 profile of subjects was relatively higher than that of an age- and gender-matched reference group of dental patients. The results suggest that the complaint of oral malodor may be related to psychopathological symptoms as recorded by the SCL-90 questionnaire.

Key words: malodor, halitosis, SCL-90 symptom survey, periodontal.

INTRODUCTION

Oral malodor (halitosis) is a common complaint and is an important reason why adults seek dental counsel (1). Many people indicate that they occasionally suffer from halitosis (on awakening or after consumption of special food), and others claim to suffer continually to the degree that it interferes with their social and professional life.

Halitosis often has a physiological etiology originating from poor oral hygiene, periodontal diseases, dry mouth, food impaction, improper or faulty restorations, unclean dentures, excessive bacterial growth on the dorsum of the tongue, nasal infections and obstructions, throat infections, and oral carcinomas (1, 2). A simple and rapid technique of measuring volatile sulfides has recently been shown to correlate with oral odor assessments by human odor judges (3, 4).

As with other human perceptions (sight, hearing, pain, etc), smell is subjective and is affected by emotional and cognitive variables. The role of the olfactory system plays a significant role in memory and cognition (5). Humans possess an acute sense of detecting and distinguishing odorants, but identifying particular odorants may sometimes be difficult (6). Smell is strongly associated with various affective states in the way that it can influence and be influenced by mood (7).

Pryse-Phillips (8) described an "olfactory reference syndrome" in which the patient claims to actually perceive a malodor that others cannot detect. The personalities of patients suffering from the syndrome show tendencies toward self-observation, self-criticism, neuroticism, inferiority, shyness, difficulty in expressing emotion, and obsessional features. Depression, when present, is usually secondary to the syndrome. One type of olfactory reference syndrome, with most of the characteristics described by Pryse-Phillips (8), is delusional halitosis. Patients who suffer from delusional halitosis have been reported in the literature (9–11).

In a study by Iwakura et al. (12), most of the patients with primary complaints of halitosis were self-conscious or became conscious of halitosis because of the indication of others. The authors suggest that most of the patients suffered from imaginary halitosis due to presumptions based on another's attitude.

The subjective and objective factors that initiate the complaint of oral malodor are not, as yet, fully understood. They may originate from awareness to the problem because of another's attitude, personal sensitivity, or both. In the present study, clinical evaluation and a psychopathological symptom survey were carried out on 38 patients who complained of oral malodor.

MATERIALS AND METHODS

The study was comprised of 38 subjects (66% females; mean age 43 ± 10 years) with a specific complaint of oral malodor who

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arrived on their own initiative to a multidisciplinary team formed to evaluate the problem (Group A). Three major complaint patterns were presented: those who perceived themselves as having a halitosis problem ($N = 16$); those who claimed that they have been told by others that they have a halitosis problem ($N = 12$); and those who both perceived themselves as suffering from halitosis and claimed to have been told by others to have the problem ($N = 10$).

On the day of the examination, the participants were instructed to refrain from using scenting personal products. Subjects were also requested to refrain from oral activities, including drinking, eating, chewing gum, and mouth rinsing, 2 hours before the appointment.

In many cases, the origin of bad breath originates from poor oral hygiene and periodontal disease, which lead to microbial putrefaction of food residues and host components (1, 13–15). Therefore, subjects underwent a clinical evaluation of their periodontal status, including recording of the following parameters: a) Plaque Index (PI) according to Silness and Loë (16), which evaluates the amount of plaque present on each tooth surface; b) Gingival Index (GI) according to Loë and Silness (17), which evaluates the severity of gingivitis; and c) Probing Depth (PD), which evaluates the depth of periodontal pockets at six reference loci of each tooth. In each case, all available sites were scored, and the mean was computed. Because of technical reasons (insufficient time), probing depth data were recorded for only 21 subjects.

The subject's oral odor was evaluated by an objective odor judge (M.R.), whose score has previously been shown to correlate with those of other judges, as well as by volatile sulfide levels in the oral cavity (3, 4). The odor judge was blinded to any other data obtained during the study. Subjects were asked to breathe out from the mouth approximately 10 cm from the nose of the judge. The judge recorded his findings on a 10-cm visual analogue scale, marked at both ends as no odor and maximal odor, respectively.

Finally, subjects were requested to score the level of bad breath that they thought they had at that time on a 10-cm visual analogue scale as described above (self-rating oral odor). To assure that subjects did not suffer from anosmia problems, they were requested to smell a sample of dung-based fertilizer and score its smell on a similar scale.

All subjects were requested to complete the SCL-90 questionnaire developed by Derogatis et al. (18) as a general measure of psychopathology in clinical and research situations. The questionnaire includes the following dimensions: somatization, obsession-compulsion, interpersonal sensitivity, depression, anxiety, hostility, phobic anxiety, paranoid ideation psychoticism, and the global scales of General Symptom Index (GSI), Positive Symptom Total (PST), and Positive Distress Index (PSDI). The SCL-90 has shown high rates of validity and reliability in numerous clinical studies (19) and has been successfully used in clinical dental settings to identify psychometric profiles of craniomandibular pain patients (20, 21) to predict the success of behavior therapy in the treatment of dental fear (22) and to evaluate patients suffering from burning mouth syndrome (23, 24).

A reference group (Group B) consisted of 30 generally healthy subjects, age- and gender-matched, who were under prosthodontic treatment in an affiliated clinic (57% females; mean age 46 ± 8 years). No subject presented a complaint of oral malodor. The reference group was requested to complete the SCL-90 questionnaire but was not submitted to further periodontal or malodor evaluations.

RESULTS

Initially, subjects with a halitosis complaint were grouped according to the type of complaint that initiated their referral for halitosis evaluation (perceived themselves as suffering from the problem, were told by others, or both). No significant differences were found between the groups in their periodontal status (PI, GI, PD; analysis of variance (ANOVA)) and/or psychopathological profiles as evaluated by the SCL-90 questionnaire (multivariate ANOVA (MANOVA)). Further evaluation was, therefore, carried out on all subjects as one group (Group A).

The mean periodontal findings for Group A were PI = 1.22 ± 0.50; GI = 1.14 ± 0.41; and PD = 2.94 ± 0.65 mm. Subjects showed a fair olfactory ability as shown by the scores given to the fertilizer sample, similar to the scores given by the odor judge (8.77 ± 1.11 on a 0–10 continuous scale). Their self-rating of oral odor was 6.69 ± 2.74 (on a 0–10 scale) and was significantly higher than the odor evaluations given by the objective odor judge (2.67 ± 1.35 on a 0–10 scale; paired samples t test, $t = -7.94$, $p < .0001$). Spearman correlation coefficients showed that all periodontal parameters were significantly associated with the odor judge measurements (PD, $r = .63$, $p = .0001$; GI, $r = .47$, $p = .001$; PI, $r = .31$, $p = .02$).

No significant correlations were found between periodontal parameters and the patients' self-rating oral odor. There were significant correlations between the self-rating oral odor scores and some of the SCL-90 scales: obsession-compulsion ($r = .27$, $p < .05$); depression ($r = .38$, $p < .01$); anxiety ($r = .34$, $p < .05$); phobic anxiety ($r = .32$, $p < .03$); paranoid ideation ($r = .27$, $p < .05$); psychoticism ($r = .27$, $p < .05$); GSI ($r = .34$, $p < .03$), and PST ($r = .28$, $p < .05$). The significantly higher self-rating oral odor scores compared with the objective odor judge's evaluations and the positive association between self-rating oral odor and eight (out of 12) of the SCL-90 scales led us to the assumption that patients who present a specific complaint of halitosis may show an elevated SCL-90 profile. When data regarding the SCL-90 profile of Group A were compared with an age- and gender-matched reference group (Group B), a general trend for an elevated profile was evident for Group A (Table 1). Significant differences were found in the subscales of interpersonal sensitivity and obsession-compulsion and the global score of PST.

TABLE 1. Psychopathological Profile of Patients with a Halitosis Complaint vs. Control Group of General Dental Patients

SCL-90	Halitosis (A)	Control (B)	p^a
Subscales			
Somatization	0.35 ± 0.36	0.31 ± 0.43	.67
Obsession	0.73 ± 0.62	0.41 ± 0.61	.05*
Personal sensitivity	0.82 ± 0.80	0.37 ± 0.58	.01**
Depression	0.60 ± 0.69	0.36 ± 0.50	.13
Anxiety	0.49 ± 0.42	0.28 ± 0.42	.06
Hostility	0.42 ± 0.40	0.28 ± 0.60	.29
Phobia	0.15 ± 0.24	0.08 ± 0.24	.27
Paranoid ideation	0.55 ± 0.75	0.44 ± 0.55	.49
Psychoticism	0.41 ± 0.61	0.17 ± 0.32	.06
Global scores			
GSI	0.51 ± 0.45	0.29 ± 0.42	.06
PST	27.90 ± 18.50	16.00 ± 15.80	.01**
PSDI	1.50 ± 0.49	1.62 ± 0.67	.45

[a] Two-tailed probability.
* Significant at the .05 level; ** significant at the .01 level.

DISCUSSION

The subjective perception of oral malodor is a complex issue. The data suggest that patients who arrive with a complaint of halitosis are more sensitive to their own odor than an objective odor judge. First, the subjective self-rating of oral odor is significantly higher than the evaluation of the odor judge. Second, whereas objective odor evaluations, as carried out by the judge, are positively correlated with the patient's periodontal status, no such correlations were found between the periodontal parameters and the subjects' self-rating. The patients' evaluations of the control sample (fertilizer) did not differ from that of the odor judge, so it seems that the reason for the high subjective oral malodor evaluations was not due to physiological predisposition. Apparently, subjects tended to score their mouth odors according to subjective, preconceived notions rather than according to objective findings.

When the individuals with a complaint of halitosis were compared with a general population (Group B), they showed relative elevations in the global scale of PST and the subscales of interpersonal sensitivity and obsession-compulsion. Although the differences between populations were not highly significant (possibly due to the small sample size), they indicate a general trend that suggests the nature of the studied population. The higher scores on the subscale of interpersonal-sensitivity suggest that the "halitosis complainers" are characterized by an increased focus on feelings of personal inadequacy and inferiority, particularly in comparison with other individuals. It may be feelings of self-consciousness and negative expectations regarding interpersonal communications that lead to the interpretation of other peoples' actions (e.g., hand or face movement when coming close to them, etc.) (12) as a reaction to their own "bad breath." The lack of significant differences between those who perceived themselves as having halitosis and those who claimed that their consciousness originated in the remarks of others further suggests that it was personal sensitivity rather than objective findings that led to the complaint.

The irresistible thoughts, impulses, and actions characteristic of persons showing high scores of obsessive-compulsive behavior may indicate an increased involvement with personal hygiene in general and with oral odors in particular. The fact that (contrary to our expectations) no increase was evident in the subscale of somatization suggests that the complaint of malodor may not be part of a broader somatoform disorder but is perhaps a more specific syndrome.

The complaint of oral malodor present a complex issue that has not received adequate attention from the professional community. Some people who suffer from repellent body or oral odor may be unaware even when the problem is pointed out by significant others. Others may be overanxious about having a potential repelling smell to the point of development of a delusion of bad smell (8, 9). In the present study, clinical psychiatric diagnostic examinations were not carried out, so one cannot know which patients, if any, had delusions and/or psychotic or mood disorders.

A most intriguing problem regarding bad breath is the apparent inability of not knowing whether one has it and to what extent (9, 10). The life of these patients who believe (rightfully or not) that they suffer from oral malodor is often strongly affected (2, 9). The inability to be sure whether or not halitosis is present makes them very unhappy (10).

Iwakura et al. (12) suggest the use of personality tests together with sensory and instrumental examinations to evaluate the complaint of halitosis. Recently, new instrumental approaches have been suggested to measure bad breath. The use of a portable monitor that measures volatile sulfide compounds in the oral cavity to evaluate halitosis was suggested by Rosenberg et al. (3, 4). Goldberg et al. (13) have recently shown that cadaverine levels in saliva are associated with odor judge scores and improve the correlation obtained with sulfides alone. Further improvement of the correlation between sulfide measurements and oral judge scores can be obtained with the use of a simple color test that records the

presence of trypsin-like enzymatic activity associated with several putative periodontopathogenic oral species (BANA test) (25).

The ability to present objective features of oral malodor in the clinic may enable their incorporation in biofeedback treatment. This may especially be beneficial to those patients whose complaint is not based on objective clinical findings. In either case, the possibility that subjective, psychological factors may play a role in the complaint should not be overlooked.

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CLINICAL ASSESSMENT OF BAD BREATH: CURRENT CONCEPTS

MEL ROSENBERG, PH.D.

ABSTRACT

Bad breath typically originates in the mouth, often from the back of the tongue. Nasal problems also can cause bad breath; odor generated in this manner can be easily distinguished from mouth odor by comparing the odor exiting the mouth and nose. In most cases, good professional oral care combined with a daily regimen of oral hygiene—including interdental cleaning, deep tongue cleaning and optional use of an efficacious mouthrinse—will lead to improvement. This article discusses common causes of oral malodor as well as methods to assess the extent of the problem.

It is likely that the majority of adults suffer from bad breath at least occasionally.[1] Since bad breath usually comes from the mouth itself, the dentist should be the first professional whom individuals turn to for help.[2] In recent years, there has been a growing awareness that bad breath is a problem that the dental profession should recognize and address. Hundreds—perhaps thousands—of U.S. dentists currently advertise breath freshening as part of their clinical services. Dentists and their staff members also may suffer from this problem, to the dismay of their patients.[2]

Together with the growing interest of dental practitioners in bad breath diagnosis and treatment, there has been an increase in research activity in this field, reflected in two recent international workshops dedicated solely to this subject (First International Workshop on Oral Malodor, Herzliya, Israel, April 1993; Second World Workshop on Oral Malodor, Leuven, Belgium, October 1995).

This article summarizes general concepts related to bad breath. Since the main clinical challenges for the practitioner are deciding whether a given complaint of bad breath is justified and whether the odor originates in the mouth or elsewhere, this article emphasizes diagnostic modalities.

HISTORY AND FOLKLORE

Bad breath has been recorded in the literature for thousands of years. The problem is discussed at length in the Jewish Talmud,[3] as well as by Greek and Roman writers.[4] Islam also stresses fresh breath in the context of good oral hygiene. The prophet Mohammed is said to have thrown a congregant from the mosque for having the smell of garlic on his breath.[3]

Ancient folk remedies abound which are still in use. In the Bible, the book of Genesis mentions ladanum (mastic), a resin derived from the *Pistacia lentiscus* tree (Figure 1) which has been used in Mediterranean countries for breath freshening for thousands of years. Other folk cures include parsley (Italy), cloves (Iraq), guava

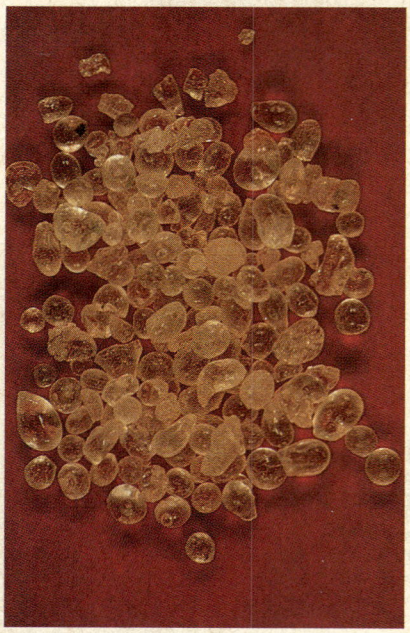

Figure 1. Gum mastic, an ancient Mediterranean remedy for bad breath, is still chewed for this purpose.

peels (Thailand) and eggshells (China).[5]

Modern literature on bad breath dates back to a monograph published by Howe in the 19th century.[6] Experimental research on the subject dates back more than 60 years.[7,8] Since the 1960s, the preeminent researcher in this field has been Dr. Joseph Tonzetich of the University of British Columbia. Among other findings, Tonzetich and co-workers established that oral malodor is associated with the presence of volatile sulfur compounds, primarily hydrogen sulfide and methylmercaptan.[1]

THE ROLE OF THE ORAL CAVITY

In as many as 85 percent of patients with bad breath, the odor originates in the mouth.[1-3,6,8,9] Several indications can suggest that the problem arises from the mouth (Table 1). The simplest way to distinguish oral from non-oral etiologies is to compare the smell coming from the patient's mouth with that exiting the nose. If the odor is primarily from the mouth, an oral origin may be inferred.[7]

In people with rigorous oral hygiene, clean and intact dentition and a healthy periodontium, the source of bad breath is likely to be the back of the tongue.[7,9] Although the anterior part of the tongue usually smells (a simple test is to lick one's wrist, waiting a few seconds until it dries, and then smell the area[6]), the main source of odor is usually farther back in the posterior region.[7] The posterior area of the dorsal surface can be readily assessed by a gentle but thorough scraping using a disposable plastic spoon.[9] Afterward, the spoon can be smelled to compare the odor with the overall mouth odor.

In many cases, a yellowish discharge is collected on the spoon (Figure 2). Although there is no direct evidence, this discharge is probably postnasal drip.[9] Postnasal drip is extremely common and may not be indicative of any frank nasal infection or other pathological condition.[6] Although the postnasal drip might not smell when it first reaches the tongue (M. Littner, D.M.D., and M. Rosenberg, Ph.D., unpublished data, 1994), it can subsequently be putrefied by the abundant tongue microbiota.

In patients with periodontal disease, the tongue has been reported to be even more malodorous.[10] Several studies have documented the presence of three potential periodontopathogens *(Treponema denticola, Porphyromonas gingivalis, and Bacteroides forsythus)* on the tongue dorsum, using the BANA test (Knowell Therapeutic Technologies, Inc.).[11-13] Data from these studies suggested that these pathogens are significantly associated with the level of whole mouth odor. Odor from the posterior dorsal surface of the tongue even may be present in preschool children.[14]

As with other body odors, bad breath usually is generated by bacteria. Putrefaction is thought to occur under anaerobic conditions, involving a range of gram-negative microorganisms, including species of Fusobacterium, Haemophilus, Veillonella, *T. denticola* and *P. gingivalis*.[15,16] Although gram-positive bacteria usually do not produce odor under laboratory conditions,[15] one research group has recently suggested that proteolysis by gram-positive *Stomatococcus mucilaginus* contributes to subsequent malodor at the dorsal tongue.[17]

Any oral site in which microbial accumulation and putrefac-

TABLE 1

Indications that Bad Breath Comes from the Patient's Mouth

- Odor exits primarily from the mouth, not the nose
- Odor is reduced following rinsing for a week with a potent mouthwash
- Odor gets worse when patient starts talking
- Odor improves following proper oral hygiene + deep tongue brushing
- Odor gets worse when the mouth dries out

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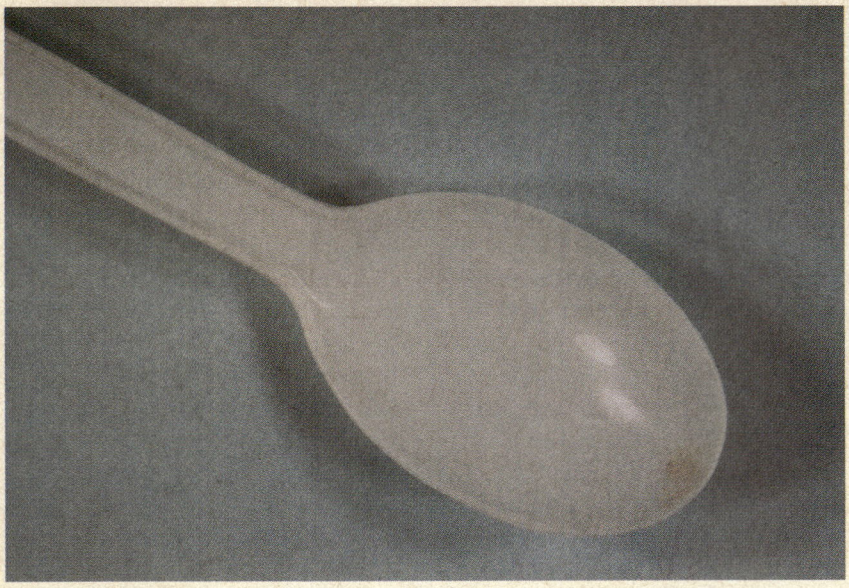


Figure 2. A simple test for diagnosing oral malodor involves scraping the posterior part of the tongue dorsum with a plastic spoon. Odor from the spoon often closely resembles the overall mouth odor and is frequently accompanied by a yellowish discharge, probably post-nasal drip.

tion can occur are suspect.[1] In addition to the most common intraoral sites of malodor production (the tongue, interdental and subgingival areas) other foci may include faulty restorations (such as overhanging restorations and leaking crowns), sites of food impaction and abscesses. Caries usually is not particularly malodorous, unless large enough to entrap food.[2]

Dentures are another important cause of oral malodor, particularly if they are worn overnight. Usually the odor has a somewhat sweet but unpleasant character and is readily identifiable,[9] especially if the dentures are placed in a plastic bag and smelled following several minutes (Y. Kaufman, D.M.D., M. Rosenberg, Ph.D., unpublished data, 1988).

Saliva also affects bad breath. In the individual, bad breath levels during the day are inversely related to saliva flow.[18,19] When saliva flow is lowest (for example, during sleep or after fasting), bad breath increases. Conversely, mastication increases saliva flow, with concomitant cleansing of the oral cavity and reduction in malodor.[1,2,19] Despite these common observations, the data of two clinical studies did not support any association between salivary flow rate and malodor levels in the populations studied.[20,21] Moreover, patients with xerostomia do not seem to have higher-than-normal oral malodor levels (L. Sreebny, D.D.S., Ph.D., personal communication, 1995). One possible explanation could be that malodor arises primarily in an alkaline microenvironment,[15] whereas the saliva of people with xerostomia often is acidic (L. Sreebny, D.D.S., Ph.D., personal communication, 1995). Additional studies should be carried out to address this apparent paradox.

When the oral etiology of the odor can not be identified, the patient can rinse and gargle for a week with a potent antibacterial mouthrinse (such as chlorhexidine-containing mouthrinses, which have been shown in several studies[20-22] to reduce odor levels significantly [$P < .001$] for long periods following use). If malodor is significantly reduced with this regimen, then an oral origin may be inferred.

EXTRAORAL SOURCES OF ODOR

Among non-oral etiologies of bad breath, the nasal passages predominate. In such cases, the telltale odor can be smelled most strongly from the nose, rather than the mouth. Nasal odor may be indicative of a frank nasal infection, such as sinusitis, or a problem affecting airflow or mucous secretions, such as polyps. In some cases, craniofacial anomalies, such as cleft palate, may be involved.[23] The tendency of very young children to insert foreign bodies into their nostrils is a common cause of offensive odor that comes from the nose yet appears to emanate from the entire body.[9] Typical nasal malodor usually has a slightly cheesy character and differs appreciably from other types of bad breath.[9]

The role of tonsils in chronic bad breath is not at all clear. A transient odor associated with tonsil infections in children is common. Occasionally, tonsils emit a foul-smelling exudate when pressed, although they appear normal on visual examination.[23] In some individuals, the tonsillar crypts develop concretions called tonsilloliths (Figure 3) which migrate to the tongue's surface. These stones are usually several millimeters in diameter, rough-edged and white or yellowish. Although

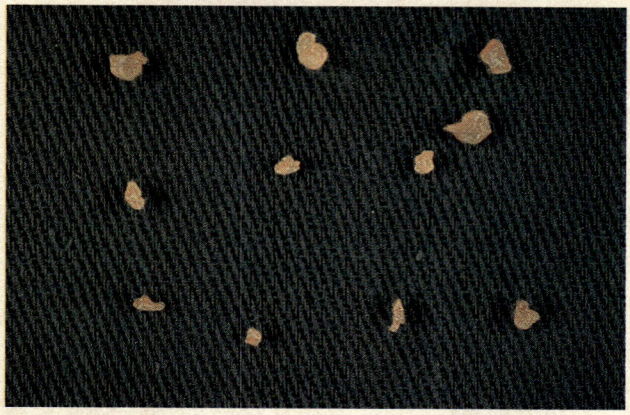


Figure 3. An assortment of tonsilloliths collected from a single patient.

the stones themselves have a foul odor, particularly when pressed, they do not appear to be a significant source of bad breath.[9]

Many non-oral diseases, including bronchial and lung infections, kidney failure, various carcinomas, metabolic dysfunctions and biochemical disorders, can result in bad breath,[24] but all these diseases taken together affect a very small percentage of people experiencing oral malodor. One interesting rare metabolic condition that leads to a perception on the part of the patient of a foul fishy odor or taste is trimethylaminuria.[25] An acetone smell to the breath was once considered a diagnostic indication for uncontrolled diabetes, but very few cases are detected in this manner (C. Hollenberg, M.D., personal communication, 1990).

Contrary to common thinking, bad breath originating in the gastrointestinal tract is considered to be extremely rare.[2,7-9,26] The esophagus is normally collapsed and closed, and although the occasional belch may carry odor up from the stomach, the possibility of air escaping continuously is remote.

Interestingly, people have smoked cigarettes for more than a hundred years to mask their bad breath,[7,25] a practice that creates an odor in its own right. Cigarette odor can linger for more than a day after smoking. In some cases, one can detect the odor of cigarette smoke on the breath of people who do not smoke but are continually exposed to the smoke of others (M. Rosenberg, Ph.D., unpublished data, 1995).

THE BAD BREATH PARADOX

People often are unaware of their own bad breath.[2,3,8,9] Our inability to smell our own oral malodor has been attributed to adaptation or dulling of sensation resulting from continual exposure.[27]

We recently addressed this question in a study in which 52 people attempted to score the level of the malodor coming from their whole mouth, dorsal tongue and saliva.[28] In the first two instances, participants appeared completely unable to score their own smells objectively. In the case of saliva, some objectivity was evident. In all cases, participants tended to score their own malodors according to preconceived notions. Based on the results of this study, it appears although the participants had the physiological capability of smelling their own oral odors (shown by their semiobjective scoring of their own saliva malodor), their perception of how bad their breath should smell confounded attempts at objectivity. Interestingly, in another study, self-estimates of bad breath by female subjects complaining of oral malodor were significantly higher than corresponding self-scores given by the male subjects ($P < .001$), whereas actual odor scores of the judge revealed that the men had significantly higher levels of bad breath than the women ($P < .001$).[9]

Whatever the underlying reasons, our insensitivity to our own bad breath can have grave consequences. People may spend their entire lives unaware that their breath is offensive. Conversely, many others suffer from halitophobia, a highly exaggerated fear that they suffer from bad breath.[3] People with halitophobia often avoid social situations and are continually preoccupied with concealing the perceived odor by frequent toothbrushing, continuous gum chewing and candy sucking, keeping a safe distance or "talking sideways."[9] They are often secretive about this fear, and in many cases, refrain from discussing the problem, even with family members. During consultation, they may even break into tears. One individual recently described the problem as a "nightmare." Another said that the fear of having bad breath "controls my soul." A third spoke of feeling "defiled."[9] In extreme cases, people with halitophobia are driven to social isolation, may have their teeth extracted and occasionally even commit suicide.[10]

What factors may predispose certain people to be overly concerned regarding bad breath? In some instances on questioning, patients may recall from childhood that someone in the immediate family (usually a parent)

suffered from bad breath. Thus, the patient upon reaching adulthood may develop a concern of having inherited this trait. Advertisements on bad breath may elicit unwarranted concerns in suggestible individuals. Others may notice a bad taste in their mouth and assume (correctly or incorrectly) that it must be related to bad breath. Some patients recall having been told once in their distant past that they had bad breath and have continued to worry about it ever since. Finally, as mentioned above, individuals with malodorous tonsilloliths may falsely infer that they have awful breath.[9]

CLINICAL DIAGNOSIS

Complaints of bad breath should be taken seriously by the dental practitioner, regardless of whether they appear justified.[2] When a patient expresses concern about his or her breath, it is important to set up a special appointment for an assessment. The patient should be instructed to refrain from drinking, eating, chewing, rinsing, gargling and smoking for at least two hours before the appointment. Patients also should avoid using scented lipstick, aftershave and perfume on the day of the consultation. Malodor examinations should not be performed on patients taking antibiotics.[2]

The initial challenge is to determine whether the patient's complaint is well-founded or has been blown out of proportion. Although a personal and medical history can provide important clues (for example, allergies, sinusitis, mouth breathing, polyps), self-reports of bad breath are notoriously subjective. For example, among 88 women who complained of bad breath, 25 rated their own breath as a 5 ("the most foul odor imaginable") on a scale of 0 to 5, although corresponding judge scores ranged from 0 to 3.5.[9] For this reason, it is important to ask the patient to bring along a confidant, usually a caring spouse or other family member. The confidant can provide a more objective picture of whether the patient actually suffers from bad breath and to what extent. Also, since bad breath can vary with the time of day (as an inverse function of saliva flow, which drastically decreases at night) and day of the month (worsening immediately prior to and during menstruation),[29] the odor present at the consultation may or may not be similar to the odor at other times. The confidant can help determine whether the odor present at the time of the consultation is typical in quality and intensity to the odor which is generally present.[9]

INSTRUMENTAL ANALYSIS

Bad breath has been attributed to volatile sulphur compounds, primarily hydrogen sulfide and methylmercaptan.[1] The level of intraoral VSC can be estimated chairside, using portable sulfide monitors (Interscan Corp.).[2] Several studies have shown that sulfide monitor readings are sensitive to reductions in mouth odor levels following use of efficacious mouthrinses.[18-20] Associations comparing sulfide monitor results and odor scores recorded by judges are significant (coefficient correlations ranging from about 0.45 to 0.65, $P < .001$ in various studies) and are relatively reproducible.[3]

Additional tests may further improve the association between sulfide monitor levels and odor judge scores. For example, in two studies, the association between volatile sulfur levels (as determined by the sulfide monitor) and odor judge scores was improved when BANA test results were factored into the regression analysis.[12,13] In a third investigation, BANA scores were associated with floss odor (scored by an investigator after proximal dental floss passage) ($P = .001$) and were reduced after chlorhexidine rinsing.[20] Although such quantitative and semiquantitative measurements can assist in testing for improvement following treatment, they do not obviate the need for the clinician to actually smell the breath emanating from the patient's mouth, nose and tongue.

In an influential study published in 1967, Tonzetich and coworkers argued that bad breath derives exclusively from sulfur-containing volatiles.[30] They based this on the observation that other volatiles tested did not escape from the saliva into the air. However, Kleinberg and Codipilly have recently shown that when the skin dries out, non-sulfur–containing gases, such as cadaverine, putrescine, skatole, indole, butyric acid and isovaleric acid, can be released over time.[15] Similarly, the malodor of an extract of putrefied saliva placed on the skin lingers for more than two hours (M. Rosenberg, Ph.D., O. Ilan, M.Sc., unpublished observation, 1995). The implication is that when saliva dries out on oral surfaces, a range of VSC and other volatiles are released. This is in agreement with the observation that bad breath increases when the mouth is dry.[18,19]

TABLE 2

Major Types of Bad Breath

- Tongue odor
- Subgingival plaque odor
- Nasal odor
- Denture odor
- Smokers' breath

Results in our laboratory show correlations between salivary levels of cadaverine and oral malodors.[31] When sulfide monitor scores and salivary cadaverine levels are both considered, the correlation with odor judge scores increases significantly.[31] Unfortunately, cadaverine is difficult to assess in the dental clinic. However, as mentioned above, BANA testing of samples obtained from various intraoral sites[12,13,21] and perhaps other tests such as the Oratest—which was the only test other than volatile sulfide levels to show significant associations with bad breath in children[14]—can be useful. Someday in the not-too-distant future, electronic "noses" that distinguish between different kinds of smells may be available for bad breath testing. Eventually, individuals may be able to breathe into their telephone mouthpiece or other device and receive an "on-line" breath analysis.

For now, clinicians interested in diagnosing bad breath must still rely on their noses to distinguish the main types of oral odors (Table 2). These include

- odor from the posterior tongue dorsum (can easily be recognized by smelling a spoon after scraping the tongue);
- periodontal-type odor (all too familiar to practicing dentists), which usually comes from subgingival areas and interdental spaces;
- characteristic nasal odor (which can be easily identified on the breath exiting the nose);
- denture odor (readily smelled by placing the dentures a plastic bag for several minutes);
- smoker's breath.

With practice and experience, these odors become distinct and recognizable, even when found in various combinations.

In some cases, although little odor appears on the breath when the patient breathes out through the mouth, the odor becomes evident when the patient starts speaking (M. Rosenberg, Ph.D., E. Leib, D.M.D., unpublished observation, 1995). Thus, in addition to asking the subject to breathe out through the mouth and nose, we now routinely ask the patient to count out loud to 20, and we smell the breath while the patient counts.

TREATMENT

The best way to treat bad breath is to motivate patients to practice good oral hygiene and to ensure that their dentition is properly maintained.[1,2] While patients often balk at using dental floss, once the connection is made between flossing and fresh breath, compliance improves.[2] Having patients smell the floss after each use is a good way to illustrate the importance of regular flossing in improving breath odor. In one study, participants who flossed had significantly less mouth odor ($P = .016$), saliva odor ($P < .001$) and salivary cadaverine levels ($P = .011$) than those who did not.[32] Furthermore, one year after the initial oral malodor examination, the percentage of participants who flossed their teeth rose from 31 percent to 65 percent.[33] Other interdental cleaners (such as toothpicks) also can be effective in identifying and cleaning sites of odor production.

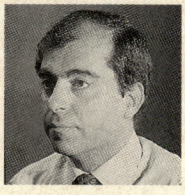
Gentle but effective deep tongue cleaning should become a part of the daily hygiene routine. Dental product manufacturers have developed a variety of tongue scrapers in recent years (Figure 4). Cleaning the tongue also can be performed by brushing with any toothbrush that minimizes gagging reflex. To prevent tongue odor, the tongue should be cleaned in a gentle but thorough manner. The patient should be reminded that the posterior portion of the tongue is the least accessible but usually smells the worst.[7-9] Even patients with significant gagging reflex can get used to cleaning the back of the tongue with some practice.

Since bad breath is worse when the mouth dries out (for example, at night or when fasting), patients should be encouraged to drink ample amounts of water. Chewing gum also is helpful in reducing bad breath during the day, although it should be chewed for only a few minutes at a time to avoid temporomandibular joint problems.[2]

Many people continue to have malodor of oral origin, even after maintaining good oral hygiene. In such instances, rinsing and gargling with an ef-

ficacious mouthwash may be advised. The best time to use a mouthwash is probably before bedtime, since residue of the mouthrinse may remain in the mouth for a longer period and have a greater effect, and bacterial activity leading to bad breath is greatest at night, when saliva flow is lowest.[1] Patients should be advised, however, that many mouthwashes contain components that may have a non-beneficial effect on soft oral tissues (including alcohol, sodium dodecyl sulfate, strong oxidizing agents).

Although consultation and treatment may result in a dramatic reduction in bad breath, patients may find it difficult to sense the improvement themselves (A. Fleming, Ph.D., personal communication, 1990). This problem can be addressed with the help of the confidant, who can help monitor changes over time. In those cases in which the odor (or the subjective complaint) persists, the patient should be referred to a physician for further assessment.



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BEING FRANK ABOUT BAD BREATH

Should a dentist tell a patient with bad breath that he or she suffers from the problem, even when the patient does not complain? Discussing this observation is an onerous but important task requiring sensitivity and tact. One possibility is to make an oblique, generalized comment about the connection between oral hygiene and bad breath. For example, the dental professional might ask, "Did you know that people who don't floss their teeth daily may suffer from bad breath?" The patient might then ask, "Are you implying that I have bad breath?" The dentist or hygienist could then respond, "If you don't clean your teeth and tongue properly, you might."

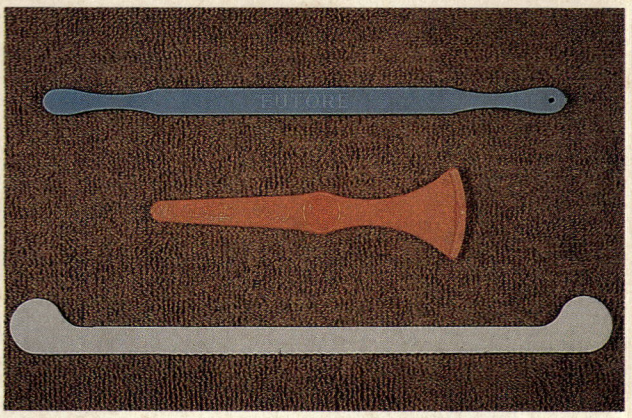

Figure 4. A variety of tongue scrapers are currently available for cleaning the tongue dorsum.

Certainly, it is difficult to tell patients, friends and associates that they suffer from this problem. However, at the very least, we should be prepared to tell the ones we love and care for if they suffer from bad breath, and to hope that, if necessary, they will reciprocate in kind.

SUMMARY

Several of the central concepts in the diagnosis and treatment of oral malodor are as follows:

- Bad breath is a common condition that usually originates in the mouth itself and rarely comes from the gastrointestinal tract.
- The dentist has the primary responsibility for diagnosing and treating bad breath.
- The posterior area of the dorsal tongue is the most frequently overlooked source of oral malodor. It can be sampled by scraping the surface with a plastic spoon.
- Patients complaining of bad breath should be assigned a separate appointment and should be encouraged to bring along a confidant—a family member or a close friend.
- Because of the difficulty inherent in smelling our own breath, many people harbor grossly exaggerated concerns about their breath, while others remain unaware that they suffer from halitosis.
- Although correlative, quantitative measurement techniques are available and helpful, the clinician also should make a differential judgment by actually smelling the odor emanating from the patient's mouth and nose.
- In most cases, bad breath can be ameliorated by proper dental care, oral hygiene, deep tongue cleaning and, if necessary, rinsing with an effective mouthwash.
- If the problem (or the patient's perception of the problem) persists, the patient should be referred promptly for appropriate medical care. ■

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Tel Aviv University's home page posts notices about research related to bad breath at "http://www.tau.ac.il/~melrose/Welcome.html".

The author thanks Rellu Samuel for assistance in the photography of the figures.

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Current events

Oral bacteria in Miocene *Sivapithecus*

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In human, as well as other animals studied to date, the interrelationship between host and associated resident microbiota plays a critical role in health and disease. The complexity of such interactions is most evident in the human mouth, which can play host to as many as 500 different adherent microbial species (Moore & Moore, 1994). Whereas in some cases the resident oral microbiota play a protective role (e.g., in preventing colonization and

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proliferation of candida species), oral bacteria are also involved in two of the most prevalent current diseases of mankind, i.e., dental caries and periodontal disease (Cataldo *et al.*, 1991; Slots & Taubman, 1992; Moore & Moore, 1994; Nisengard & Newman, 1994), and may even be linked to coronary heart disease (DeStefano *et al.*, 1993). Both these diseases are considered to be associated with colonization of the tooth surface by certain strains of particular species. Dental caries are thought to be associated with mutans streptococci, although *Actinomyces* and lactobacilli have also been considered to play a role (Cataldo *et al.*, 1991; Slots & Taubman, 1992; Nisengard & Newman, 1994). Periodontal diseases may involve a wide variety of proteolytic Gram-negative species, including *Fusobacterium*, *Porphyromonas gingivalis*, and spirochetes (Cataldo *et al.*, 1991; Slots & Taubman, 1992; Moore & Moore, 1994; Nisengard & Newman, 1994). Although evidence for both these diseases have been found in skulls of early Pleistocene hominids (Clement, 1958; Ripamonti, 1988), little is known of the ancient microbial makeup of hominoid/hominid dental plaque.

Calculus is a mineralized plaque that adheres to the tooth surface and is covered by an unmineralized bacterial layer (Gibbons & Van Houte, 1973; Caton & Quinones, 1991; White, 1991). Within the mineralized matrix bacteria are the main component with some epithelial cells, blood cells, and remnant food debris (Friskopp & Hammarstorm, 1980; Dobney & Brothwell, 1986; Linossier *et al.*, 1988; Hershkovitz *et al.*, 1997). Owing to its mineral content, dental calculus survives well in fossils (Hershkovitz *et al.*, 1997; Vandermeersch *et al.*, 1994).

In the present study, we have examined the dental calculus obtained from the fossil ape *Sivapithecus*. Specimens of this primate are known from middle to late Miocene sediments of South Asia (ca. 12·5–8·5 million years bp) (Pilbeam *et al.*, 1977). *Sivapithecus*, is generally attributed to the extant orang-utan lineage, based on a number of presumed shared derived cranial features (Andrews, 1992).

The studied specimens were collected from exposures of the Siwalik Sequence on the Potwar Plateau, Northwest Pakistan (Pilbeam *et al.*, 1982). Dental calculus was present in two of seven *Sivapithecus* specimens (some represented solely by isolated teeth): GSP (Geological Survey of Pakistan) 15000 and GSP 49567. GSP 15000 consists of a partial skull and complete mandible, recovered *in situ* from overbank sediments paleomagnetically dated to ca. 9·3 million years bp. GSP 49567 is a partial mandible recovered as a surface find from similar sediments, dated to ca. 8·7 million years bp.

Chips of *Sivapithecus* supragingival calculus were pried off the tooth surface of the lower second left molar of GSP 15000 and second molar of GSP 49567, using dental scaler (in this manner the calculus was entirely removed from tooth surface). The calculus samples were examined by scanning electron microscopy in two successive stages (Figure 1). Preliminary classification of biologic forms (e.g., bacteria) was done on the basis of shape and size (Boyde & Williams, 1971; Holt, 1984). To ease identification, stereoscopic images, obtained by tilting the stage 8° between two exposures of the same location, were obtained. The preserved microbiota in the two samples of *Sivapithecus* dental calculi were compared with calculi microbiota in Pleistocene and modern humans and in apes. Comparative samples included dental calculus (sub- and supragingival) from the second and third upper and lower molars of 15 free-ranging apes (five *Pan troglodytes*, five *Gorilla gorilla* and five *Pongo pygmaeus*) and 15 modern humans (Hamann-Todd collection, The Cleveland Museum of Natural History), one Neandertal, Spy I (Laboratoire d'Anthropologie et de Genetique humaine, Universite Libre de Bruxelles), plus 15 prehistoric (natufian) hunter–gathers (ca. 10,500–8300 B.C.) and 15 early neolithic farmers (ca. 8300–6500 B.C.) (Department of Anatomy collection, Tel Aviv University).

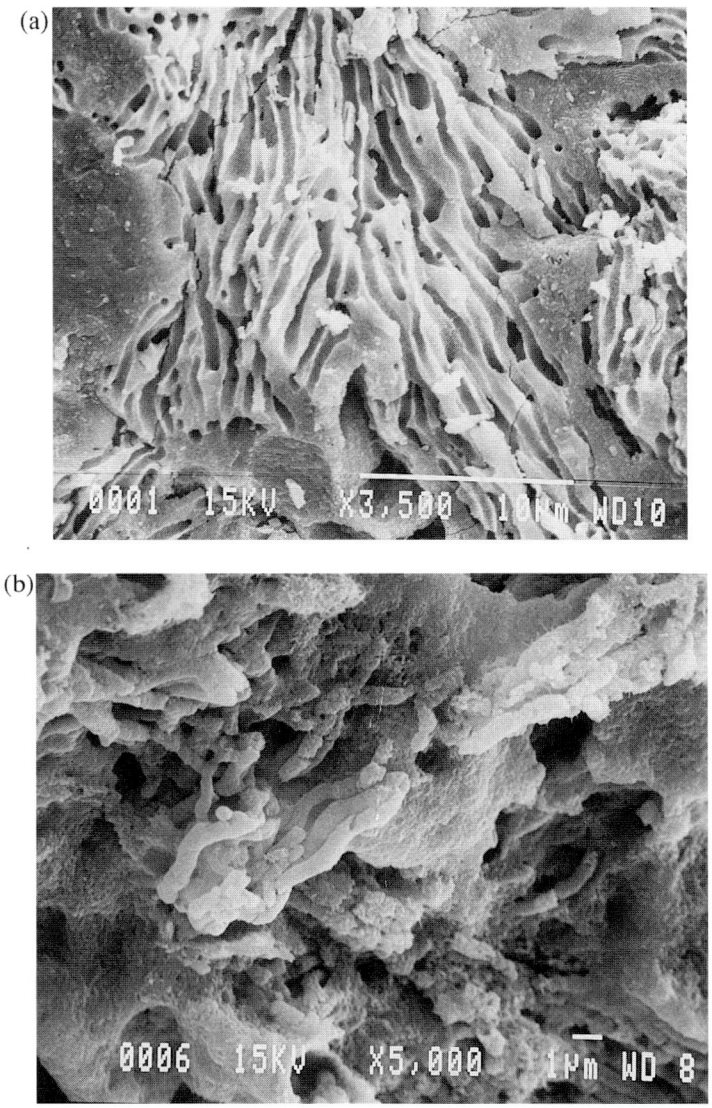

Figure 1. Scanning electron micrographs of GSP 15000 calculus. (a) Stage 1: Calculus samples were sectioned, cleaned, and affixed to an aluminum specimen mount with double sticky carbon tape so that the exposed split surface of the calculus could be examined. Specimens were coated with 20 nm palladium/gold in an RMC-Eiko IB-3 ion coater, and examined in a JOEL 840-A scanning electron microscope. Impressions of filamentous micro-organisms, appearing as shallow grooves, holes and cross-cut channels, were observed. (b) Stage 2: A few samples were removed from specimen mounts, immersed in 1N HCL for 2 min, rinsed thoroughly in distilled water, and dried in a 65°C oven for 30 min. Digested samples were gently collected with double sticky carbon tape and affixed with the calculus to an aluminum specimen mount. The samples were coated again and reexamined. Fossilized bacteria are clearly evident.

The fractured surfaces of the supragingival calculus of GSP 15000 had a smooth, solid appearance (Figure 1). Scanning electron microscopy revealed the microbial composition of the fossilized calculus to be remarkably homogeneous, with similar looking filamentous morphotypes comprising approximately 99% of the microbial fossils observed (Figure 1).

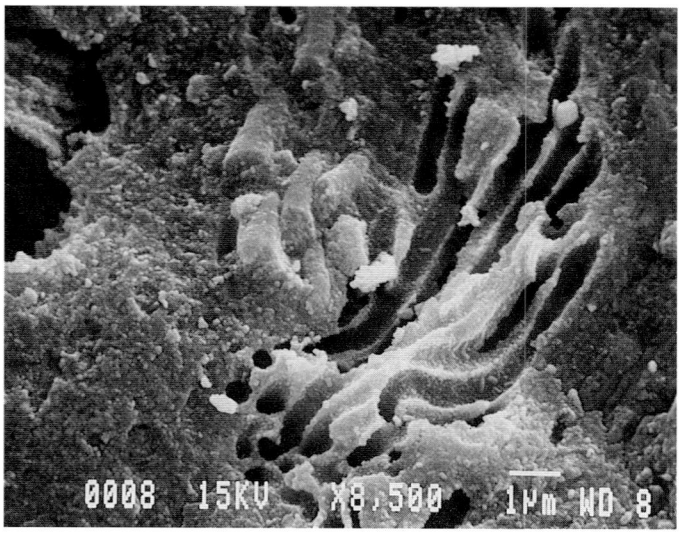

Figure 2. Scanning electron micrographs of GSP 49567 calculus. Shallow grooves are indistinguishable in appearance from those seen in Figure 1(a). Notice the fossilized bacteria adjacent to the grooves.

The filamentous bacteria, $0{\cdot}5 \pm 0{\cdot}1$ μm in diameter, appeared to be aligned and tightly packed in the center of the calculus (i.e., half way from the tooth surface to the plaque exterior), and extended outwards, in an increasingly random fashion, from 10–50 μm from the fractured matrix (being partially embedded within the matrix). Straight or slightly curved short bacilli (0·3–0·9 μm in diameter and 1·5–5·0 μm in length) and coccal forms (ca. 0·4–0·9 × 0·4–0·9 μm) were rarely observed. The matrix of GSP 49567 contained filamentous structures identical to those of GSP 15000 (Figure 2). Several isolated 1·3 μm diameter filaments were also present in this specimen. The filaments observed in the *Sivapithecus* samples are similar in size and shape to *Actinomyces* and *Nocardia*, current common residents of dental plaque. The 0·5 μm cross-section is too small for fungal filaments, and septae characteristic of algal forms were not found.

Whereas dental plaque samples from contemporary humans and apes also contain filamentous bacteria, especially in mature plaque, they appear to be much more heterogeneous in appearance and distribution (Figure 3), and share the calculus matrix with a variety of cocci and bacilli. In *Sivapithecus*, in contrast, bacilli and coccal forms were rarely observed (less than 1%).

As bacteria are an inherent component of dental calculus (Newman & Britton, 1974; Lustmann *et al.*, 1976; Listgarten, 1986; Carrassi *et al.*, 1988), observation of a filamentous form in essentially pure "culture" would seem to exclude the possibility of simple post-mortem contamination, for example by soil organisms such as *Streptomyces*. Absence of external surface invasion of the calculus by filamentous forms mitigates against the possibility that the observed structures result from post-mortem contamination. Furthermore, the cells appeared to proliferate outwards from the tooth surface, as generally observed in dental calculus (Friskopp & Hammarstorm, 1980; Nisengard & Newman, 1994), whereas a contamination would show the opposite orientation. The observed forms respect physiologic constraints (e.g., oriented predominantly perpendicular to the tooth surface) (Friskopp & Hammarstorm, 1980; Nisengard & Newman, 1994), in contrast to contaminants which permeate entire structures.

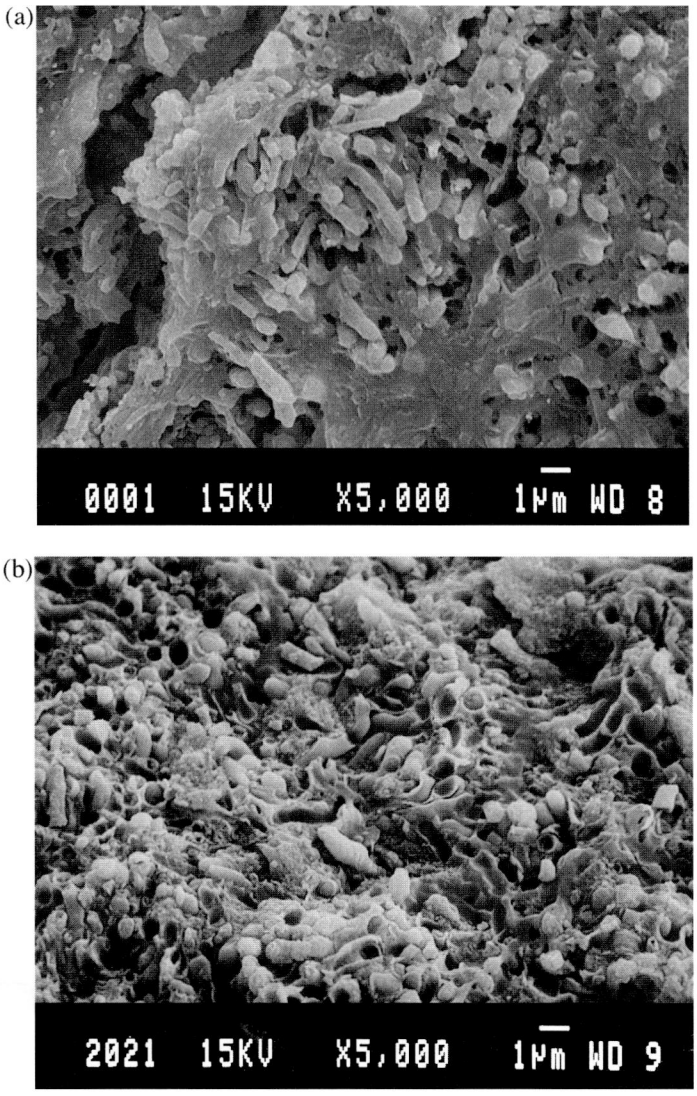

Figure 3. Bacterial colonies in chimpanzee (*Pan troglodytes*) calculus (Left B-1434; Right B-1806, Hamann-Todd collection). Note the heterogeneous nature of the bacterial types.

To our knowledge, this is the first study to observe dental micro-organisms in fossils more than 60,000 years old. In a recent study on Neandertal teeth, Vandermeersch and coworkers (Vandermeersch *et al.*, 1994) observed that the composition of bacteria in Neandertal calculus is very similar to that of modern humans. Yet, our data raise the possibility that in early hominids and hominoids, the oral microbiota was much less complex than in current humans or apes. Similarly, the increases in prevalence of both dental caries and periodontal disease observed during the last few thousand years might possibly be related to relatively sudden changes from relatively homogeneous to extremely heterogeneous dental plaque (Hershkovitz *et al.*, 1997). Whether such postulated changes may have derived from differences

in diet, social behavior, immunological considerations, or association with domesticated animals is a matter of speculation.

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Hydrophobic Interactions and Inhibition of Microbial Adhesion

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Abstract

Over the past few decades, it has become clear that hydrophobic interactions are an important factor in microbial adhesion to a wide variety of surfaces. This includes not only non-wettable surfaces, but a variety of substrata in the environment and host. Various cell surface components that promote adhesion *via* hydrophobic interactions (»hydrophobins«) or interfere (»hydrophilins«) have been identified. The ability of soluble amphipathic agents to interfere with adhesion has been tested using a variety of components and test systems. Another approach which we have used is the so-called BAWA (»beat adhesion with adhesion«) in which adhering bacteria can be desorbed from a surface by preferential adhesion onto the oil : water interface. We have successfully used oil droplets to desorb bacteria from oral surfaces, and since 1992, a proprietary twophase mouthwash has been commercially available in Israel, based on this scientific principle.

1. Introduction

Cell surface hydrophobicity has become of increasing interest during the last decade. Hydrophobic interactions play an important role in different aspects of microbial ecology. Examples include adhesion to solid surfaces, partitioning at liquid:liquid and liquid:air interfaces, nutrient scavenging, gliding and spreading, and interactions with phagocytic cells (Rosenberg and Kjelleberg 1986, Rosenberg and Doyle 1990). Medically-related microbial adhesion influenced by hydrophobic interactions include adhesion to teeth, fatty tissues, host insect cuticle, catheters, intrauterine contraceptive devices, bioprostheses, contact lenses, and other biomaterials, as well as a variety of epithelial and phagocytic cell types (for a review see Rosenberg and Doyle 1990). During the last decade, many methods were developed for assessment of cell surface hydrophobicity, each with its advantages and disadvantages (for a recent technique, see Straver and Kijne 1996). In 1980, we proposed a rapid method for assessing microbial cell surface hydrophobicity, based on adhesion to oil droplets. In this assay, washed cell suspensions are vortexed with the test liquid hydrocarbon (mainly n-hexadecane), and the degree of adhesion of cells to the oil droplets is measured spectrophotometrically following phase separation (Rosenberg et al. 1980). Although other tests for cell surface hydrophobicity have been developed, the simplicity of the so-called MATH (microbial adhesion to hydrocarbons) made it a popular assay (Bar-Ness Greenstein et al. 1995, Lin et al. 1995), but their discussion is beyond the scope of this chapter; for reviews see Rosenberg and Doyle 1990, Rosenberg et al. 1991a, van der Mei et al. 1991). The MATH test subsequently led us to seek agents which can prevent or reverse adhesion by interfering with hydrophobic interactions.

Conversely, we found ways to promote adhesion to oil droplet, leading us to develop a novel two:phase microbe-desorbing mouthrinse, based on the BAWA (»beat adhesion with adhesion«) approach.

2. Adhesion and Microbial Cell Surface Hydrophobicity

Generally, the attachment of bacteria to surfaces depends on several factors, including the properties of the substratum, the liquid phase, and the bacteria themselves (PRINGLE and FLETCHER 1986).

Biofilm formation occurs on virtually any surface in any environment in which bacteria are present. The nature of the growth environment has a profound influence on the regulatory mechanisms determining surface properties of bacteria. The gram-negative cell envelope shows remarkable flexibility in both structure and composition in response to changes in the surrounding environment (VAN LOOSDRECHT et al. 1987). ALLISSON et al. (1990) studied the possible involvement of the division cycle in dispersal of *Escherichia coli* from biofilms. They found no significant differences in surface hydrophobicity for cells grown either planktonically or as biofilms. Both populations became less hydrophobic as the growth rate was increased. Cells increased in surface hydrophobicity upon the onset of the stationary phase. These results indicate that changes in surface hydrophobicity may be associated with the division cycle and that surface hydrophobicity immediately after division is low. Hence, bacteria dislodged from these biofilms (due to low surface hydrophobicity) provided an inoculum of new cells capable of replication and colonization of new surfaces. The opposite trends of surface hydrophobicity and growth rate may imply that the changes in surface hydrophobicity reflect alterations of the cell surface with respect to surface appendages or proteins associated with adhesion. Such adhesive structures are minimized during and immediately after division, leading to daughter cell separation and dispersal (ALLISON et al. 1990).

The above trend of surface hydrophobicity and growth rate found in *E. coli* was not valid in the case of *Pseudomonas* sp. In *Pseudomonas aeruginosa* hydrophobicity increased with growth rate (VAN LOOSDRECHT et al. 1987). In *Pseudomonas aeruginosa* it was found that bacterial cell surface hydrophobicity is the major parameter influencing the kinetics of adhesion to stainless steel. The bacterial cell surface charge seemed of minor importance in this process, as seen in fifteen different isolates of this strain (VANHAECKE et al. 1990).

There are many examples of changes in surface hydrophobicity during procaryotic development, in gram-positive as well gram-negative bacteria (ROSENBERG and SAR 1990). One example is the change in cell surface hydrophobicity during the formation of spores. In *Myxococcus xanthus*, a gram-negative bacterium, sporulation was accompanied by a dramatic decrease in surface hydrophobicity (KUPFER and ZUSMAN 1984). The opposite trend was found in the gram-positive bacterium *Bacillus subtilis* and several other *Bacillus* sp. DOYLE and coworkers have reported that all *Bacillus* spores tested were more hydrophobic as compared with the corresponding vegetative cells (DOYLE et al. 1984). Another example of the influence of growth phase and growth conditions on bacterial adhesion to surfaces, is demonstrated in several gram-negative bacteria i.e., *Enterobacter cloacae*, *Pseudomonas fluorescens* and *Flexibacter* sp. The cell surface hydrophobicity of these strains varied with growth phase and with the

growth medium used. Therefore, the adhesion ability of these bacteria to polystyrene was affected as well (McEldowney and Fletcher 1986).

Growth temperature may also affect adhesion ability of bacteria to different surfaces. In *Serratia marcescens*, all strains tested were adherent following growth at 30 °C whereas none retained adherence properties following growth at 38 °C (Rosenberg et al. 1986).

The significance of the substratum properties in facilitating microbial adhesion has been studied intensively. Attachment of bacteria to surfaces has been shown to be influenced by factors such as surface charge, surface-free energy, and the presence of adsorbed macromolecules. Whenever a solid surface is immersed into an aqueous solution, solutes are quickly adsorbed onto the surface. Macromolecules are generally irreversibly adsorbed and have a subsequent effect on bacterial attachment to the surface. The hydration of a surface as well as the adsorption of macromolecules can reduce bacterial attachment. However, additional factors, relating to the chemical composition of the substratum and the stabilization of the suspended bacteria, can effect the adhesion interaction and the resulted number of attached bacteria (Pringle and Fletcher 1986).

The bacterial cell surface plays an important role in adhesion to oral surfaces, including both hard and soft tissues (van der Mei et al. 1990). As in the case of other surfaces, adhesion of oral microorganisms to mouth surfaces is governed by a complicated interplay between the properties of the adsorbed proteins on these surfaces and the characters of the surfaces themselves (Pratt-Terpstra and Busscher 1991).

In the case of bacterial adhesion to teeth, bacterial repopulation is promoted by intrageneric coadhesion (coaggregation among oral species, e.g. pairs of streptococci and actinomyces; Kolenbrander et al. 1990). Although the role of bacterial hydrophobicity is not inherently obvious in the coaggregation process, many adherent oral bacteria exhibit hydrophobic cell surfaces (Weiss et al. 1982). Also, most freshly oral isolates as well as many laboratory oral species are able to adhere to oil droplets after brief mixing periods (Ofek et al. 1983, Shah et al. 1989). Rosenberg et al. used *Porphyromonas* (*Bacteroides*) *gingivalis*-coated hexadecane droplets to study the coadhesion of *Actinomyces viscosus* with *P. gingivalis*. They suggested that initial attachment of individual *A. viscosus* cells to *P. gingivalis* cells may occur through complementary (but not lectin mediated) stereochemical associations. Subsequent attachment of additional *A. viscosus* is greatly promoted by lateral cell-cell interactions with other bound *A. viscosus* cells, in addition to attachment to the *P. gingivalis* cells. Such »like-like« associations may depend more on hydrophobic interactions. This model can account for the inhibition of coadhesion by defatted BSA, which is known to be a potent inhibitor of microbial adhesion to hydrophobic surfaces (Rosenberg et al. 1991 b).

The issue of bacterial attachment to surfaces has implications on the biocompatibility of biomaterials. Most of the research on this subject was performed on gram-positive bacteria (i.e. streptococci and staphylococci), whereas gram-negative bacteria are probable candidates for causing the most severe problems of undesirable adhesion. Several nonionic surfactants were found to prevent adhesion of a *Pseudomonas* strain and a *Serratia marcescens* strain to hydrophobic surfaces but not to hydrophilic ones (Humphries et al. 1987). This antiadhesive effect could have important applications in many areas where bacterial fouling is a major problem (Humphries et al. 1987).

3. Hydrophobins and Hydrophilins

The term »hydrophobin« was originally coined by ROSENBERG and KJELLEBERG (1986) to denote hydrophobic surface components that promote cell surface hydrophobicity and adhesion. Conversely, cell surface components that reduced hydrophobic surface properties of microbial cells were termed »hydrophilins«. In recent years use of the term hydrophobin has been extended by WESSELS and coworkers to include hydrophobic surface components of fungal cells that may or may not be involved in adhesion processes (WESSELS et al. 1991 a, b). A summary of the work done in other laboratories on hydrophobins and hydrophilins of several microbial species is presented below.

3.1 Acinetobacter calcoaceticus

Acinetobacter calcoaceticus species, although widespread in the environment, can also inhabit the human body and are sometimes associated with nosocomial infections. Our studies concentrated on strain RAG-1, a bacterium which degrades oil and has been exploited commercially. Stationary phase cells were found to exhibit a high affinity for the oil:water interface. Mutant MR-481, selected for its inability to adhere to oil droplets, was devoid of thin fimbriae present on wild type cells. Revertants that regained partial adhesion characteristics were found to possess copious thin fimbriae (ROSENBERG et al. 1982). These data suggested that the thin fimbriae function as hydrophobins of RAG-1 cells. However, another RAG-1 mutant was found to be able to adhere, albeit with somewhat lower affinity, at the oil:water interface, even though it lacked thin fimbriae. Thus, other RAG-1 hydrophobin(s) remain to be elucidated.

Emulsan is a capsular anionic heteropolysaccharide of *A. calcoaceticus* RAG-1 which is sloughed off (together with some bound protein) into the growth medium, when cells reach stationary phase. Emulsan exhibits amphipathic properties and is a potent emulsifier of oil droplets. When free in solution, emulsan acted as a potent inhibitor of microbial adhesion (see further). We originally hypothesized that it might act on the cell surface to promote adhesion. However, the opposite turned out to be the case. Cells, after having shed their emulsan, became increasingly hydrophobic. Similarly, the rhamnose-rich capsule of another strain, *Acinetobacter calcoaceticus* BD 4, although completely different in composition, also prevented adhesion to hexadecane while on the cell surface. Concomitant with enzymatic degradation of the capsule on the cells themselves, adhesion to oil droplets increased from zero to almost 100% (E. ROSENBERG et al. 1983 a, b).

3.2 Streptococcus pyogenes

Streptococcus pyogenes is a common cause of throat infections, and other, sometimes life-threatening diseases. Cells of *S. pyogenes* adhere avidly to hexadecane and other oil droplets (OFEK et al. 1983). LTA and M proteins have both been considered as promoting hydrophobic surface properties of *S. pyogenes*, both individually and in association with one another. Conversely, hyaluronate capsule appears to act as a hydrophilin, and the hydrophobic-to-hydrophilic conversion of cells may be critical for successful colonization. *S. pyogenes* cells may initially be hydrophobic, to facilitate initial adhesion, but subsequently elaborate capsule to prevent phagocytosis (OFEK et al. 1983).

3.3 Serratia marcescens

Serratia marcescens is a widespread microbial species which can be a troublesome source of nosocomial infections, particularly in neonatal wards. It has been recognized for some time that prodigiosin, the red, oil-soluble pigment characteristic of pigmented strains, imparts hydrophobic surface characteristics, enabling cells to partition at air : water interfaces (HERMANSSON et al. 1979). Nevertheless, nonpigmented clinical strains are also capable of adhering (albeit, less avidly) to nonwettable surfaces (ROSENBERG et al. 1986). Furthermore, cells grown at temperatures above 37 °C lose pigmentation, yet remain adherent. Finally, nonpigmented mutants may retain partial hydrophobic surface characteristics (ROSENBERG 1984). BAR-NESS and ROSENBERG (1989) demonstrated that a 70-kDa surface protein, serraphobin, which is released from *S. marcescens* cell surface during growth, is itself capable of binding to oil droplets. Furthermore, a nonadherent, nonhydrophobic yet pigmented mutant, was found to lack this surface protein (BAR-NESS 1992). Transformation of *Escherichia coli* with DNA fragments from *S. marcescens* resulted in a transformant clone exhibiting an increase in hydrophobic surface properties, concomitant with appearance of a *S. marcescens* antigen (as yet unidentified) on the surface of transformant cells. Thus, additional hydrophobins may also be present on *Serratia marcescens* cells (BAR-NESS GREENSTEIN et al. 1995).

Serratia marcescens shows spreading growth on various surfaces (MATSUYAMA et al. 1990). The spreading growth of this strain occurs at 30 °C, a temperature which allows production of serratamolide, a known specific aminolipid wetting agent. Mutants defective in wetting agent production showed retarded spreading growth (MATSUYAMA et al. 1989). As to the influence of cell surface hydrophobicity on spreading growth of *S. marcescens*, it still remains somewhat unclear. The amphipathic wetting agent, serratamolide, appears to act as a hydrophilin, rather than as a hydrophobin. Mutants lacking this surfactant molecule exhibited higher adhesion levels than isogenic wild type cells (BAR-NESS et al. 1988).

Recent results (BAR-NESS and ROSENBERG, unpublished data), show differences in spreading behavior between three strains of *S. marcescens* differing in wetting agent activity as well as in cell surface hydrophobicity. It seems that wetting agent production, combined with cell surface hydrophobicity, plays an important role in spreading growth of these strains. The wild type strain showed the most extensive spreading pattern as well as highest spreading rate as compared to the two mutants. These data imply that cell surface hydrophobicity may account for the increased spreading growth observed in the wild type strain (ROSENBERG and BAR-NESS, unpublished data).

In general, the possible advantage of such spreading phenomenon for these bacteria may be in facilitating the ability of adherent cells to spread out over the attachment surface (MATSUYAMA et al. 1989).

3.4 Candida albicans

Candida albicans is an opportunistic yeast-like microorganism which can cause both superficial and systemic infections. Although hydrophobic surface properties of *C. albicans* have been reported in various studies (CALDERONE and BRAUN 1991, HAZEN et al. 1991, TRONCHIN et al. 1988, 1989), the cell surface moieties involved have not been elucidated. In order to look for potential surface hydrophobin(s) of this microorganism, a genomic library of *C. albicans* in a nonadherent strain of

Saccharomyces cerevisiae was constructed (BARKI et al. 1993). Adherent transformants were selected by allowing cells harboring the candidal gene library to come in direct contact with polystyrene, followed by washing to remove nonadherent cells, and overlaying a selective agar. This selection procedure yielded a transformant which exhibited enhanced adhesion to treated and untreated polystyrene. Transformant cells were also highly autoaggregative in the presence of calcium ions, as compared to *S. cerevisiae* control cells bearing the vector alone. Analysis of this transformant revealed an 4.5 kb insert originating from *C. albicans*. Curing of the plasmid resulted in loss of adhesion and autoaggregation properties. The adhesion/autoaggregation phenotype observed in transformant cells appeared due to expression of a *C. albicans* surface antigen. Rabbit antiserum, raised against transformant *S. cerevisiae* cells and adsorbed with *S. cerevisiae* bearing the vector alone, bound to the surface of transformant *S. cerevisiae* cells as well as to *C. albicans* cells (especially to the invasion-associated mycelial phase), but only marginally to the *S. cerevisiae* control. Furthermore, adsorbed antisera specifically inhibited autoaggregation of transformant cells. Western immunoblot analysis disclosed an antigen of ca. 30 kDa molecular weight, present in transformant cell surface extracts, but absent from control (BARKI et al. 1994). The extent to which this antigen acts as an adhesin/hydrophobin on *C. albicans* cells needs to be further explored.

4. Soluble Agents that Appear to Inhibit Adhesion via their Effect on Hydrophobic Interactions

A wide variety of amphipathic substances may inhibit adhesion to animate and inanimate surfaces (for a review see ROSENBERG and DOYLE 1990). Some examples are detergents (e.g., sodium dodecyl sulfate, Tween 80, Tween 20, Triton X-100), chaotropes (e.g., thiocyanate anion), components obtained from body fluids (e.g., bovine serum albumin [particularly defatted], normal human serum, salivary components, caseinoglycopeptides) and small organic molecules (e.g., p-nitrophenol, tetramethyl urea, propanol, fatty acids).

Amphipathic components in saliva appear to act as a barrier, preventing free microorganisms from adhering to oral surfaces. Washed microorganisms obtained directly from dental plaque were capable of adhering to buccal epithelial cells *in vitro*, but this was largely inhibited when saliva was added (ROSENBERG et al. 1989). Saliva prevented microbial adhesion to polystyrene, but when the saliva was preextracted with hexadecane, it lost its inhibitory ability (ELI et al. 1989). BABU and coworkers demonstrated inhibition of adhesion of *Streptococcus sanguis* to hexadecane by specific hydrophobic proteins of saliva (BABU et al. 1986).

One interesting aspect is the inhibition by amphipathic molecules of adhesion apparently mediated by sterospecific, lectin-like interactions. Adhesion of *E. coli* to epithelial cells, while sensitive to mannose, can also be blocked by unrelated amphipathic compounds, such as p-nitrophenol (FALKOWSKI et al. 1986). *A. viscosus* cells preferentially adhered to *P. gingivalis* cells, rather than to hexadecane, yet this ostensibly specific interaction was inhibited by defatted bovine serum albumin (ROSENBERG et al. 1991 b).

The therapeutic use of antiadhesion agents in controlling microbial infection must consider that in most cases, we become aware of the intruding microorganisms after they have already adhered. In other words, for most medical purposes, antiadhesion

agents must be able to desorb already attached microorganisms, as well as to inhibit or prevent initial and subsequent adhesion. Unfortunately, prevention of initial attachment is easy, as compared with detaching microorganisms after they have already adhered, interacted with neighbouring bacteria, elaborated a capsular glycocalyx, and so on.

Relatively few studies have dealt with agents that affect microbial desorption. In one such investigation, E. ROSENBERG and coworkers were able to show that when the capsular anionic heteropolysaccharide of *A. calcoaceticus* RAG-1 (»emulsan«) is sloughed off into the aqueous phase, it is not only capable of inhibiting bacterial adhesion to buccal epithelial cells, but also desorbs a substantial fraction of the indigenous attached microbiota (E. ROSENBERG et al. 1983a, b).

Another consideration facing antiadhesion therapy is the observation that bacteria will eventually adhere to and foul practically every available interface. As Kevin C. MARSHALL succinctly remarked in this context: »The Bacteria Always Win.« In the following section, we describe an alternative antiadhesion strategy, i.e., the BAWA (»beat adhesion with adhesion«) approach. According to this strategy, adhering bacteria can be desorbed from one interface by preferential adhesion to a second interface. We have successfully used oil droplets to remove bound bacteria from oral surfaces in this manner (M. ROSENBERG et al. 1983a). It is important to emphasize that in such an instance, the mechanisms governing the initial adhesion are of secondary importance, as compared with the affinity of the bound cells for the disorbing interface.

5. Research and Development of a Bacteria-Desorbing Mouthwash

Over the past thirteen years, much of our interest in antiadhesion therapy has focused on the potential of two-phase, oil:water mixtures to remove oral bacteria and to combat bad breath. Our initial finding was that a high proportion of oral bacteria adhere to oil droplets. In 1982, NESBITT and coworkers showed that *Streptococcus sanguis* adhered to toluene droplets (NESBITT et al. 1982). In the same year, WEISS et al. reported that most oral microbial isolates retrieved from extracted teeth adhered to hexadecane. In 1983, ROSENBERG and coworkers subsequently demonstrated that washed, dispersed suspensions of microorganisms obtained directly from supragingival dental plaque adhered in high proportions to droplets of liquid hydrocarbon (ROSENBERG et al. 1983a). These initial observations led to the notion of using two-phase oil:water mouthwashes for physical desorption of oral microbiota (WEISS et al. 1985). Indeed, other studies have demonstrated that a wide variety of laboratory strains of oral microorganisms, including *Actinobacillus actinomycetemcomitans*, *Actinomyces viscosus*, *Actinomyces naeslundii*, *Porphyromonas gingivalis*, *Streptococcus salivarius*, *Streptococcus sanguis* and certain *Streptococcus mutans* strains, adhered with high affinity to hydrocarbons (for a review, see DOYLE et al. 1990). Initial experiments showed that microorganisms which adhere to hydrocarbons also bind to other nontoxic oils (ROSENBERG et al. 1983a). Furthermore, *in vitro* experiments showed that water:oil combinations could desorb bacteria from plastic surfaces (ROSENBERG et al. 1983b). Desorption could also be demonstrated *in vivo*, based on removal of erythrosine-stained bacteria and debris from the mouth following rinsing with a two phase rinse consisting of saline and olive oil (WEISS et al. 1985). Similar combinations were also able to remove bound cells from polystyrene cuvettes (GOLDBERG and ROSENBERG 1991).

Despite these initial desorption experiments, the simple aqueous:oil combinations did not prove particularly effective in improving oral hygiene over time. Furthermore, the two immiscible oil and water phases posed technical difficulties. In 1990, GOLDBERG and coworkers found that adhesion of microorganisms and oral debris to oil was promoted by low concentrations of cetylpyridinium chloride (CPC), an amphipathic cationic agent which is commonly added to mouthwashes (GOLDBERG et al. 1990a, b). This was surprising, since amphipathic agents were generally considered to inhibit adhesion at the oil:water interface. Since CPC is an antibacterial agent, its addition had several additional advantages, including inhibition of microbial activity in the mouth, and increasing shelf life of the product by preventing microbial outgrowth. Furthermore, addition of an amphipathic agent such as CPC to oil:water mixtures results in a temporary emulsion following shaking. This in turn enabled the mixture to be supplied in a single bottle.

Prototype formulations, consisting of an aqueous phase containing CPC, and an oil phase comprising a mixture of vegetable and essential oils, were subsequently developed and tested. Such formulations maintained dramatic *in vitro* bacterial desorption properties (GOLDBERG and ROSENBERG 1991), removing over 90% of a bacterial film of *A. calcoaceticus* RAG-1 on polystyrene cuvettes, as compared to 15–50% removal for a series of commercial mouthwashes.

In 1992, the prototype two-phase oil-water mouthwash was reformulated and introduced in the Israeli market. A clinical study (KOZLOVSKY et al. 1996) was performed to compare the efficacy of the commercial version of the two-phase mouthwash with Listerine™, a mouthrinse which has been previously shown (PITTS et al. 1983, DEPAOLA et al. 1989) to be effective in reducing levels of odor-related microorganisms, as well as plaque and gingivitis.

To our knowledge, this is the first modern antiadhesion mouthrinse developed based on adsorption of microorganisms onto an alternative surface. It is however, noteworthy, that the Jewish Talmud (Bavli, Masechet Yoma, page 84), written over two thousand years ago, prescribes a mouthrinse comprising of olive oil, salt and dough water for treating oral ailments.

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Reduction of Oral Malodor by Oxidizing Lozenges*

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THE MAIN PURPOSE OF THE STUDY was to examine the anti-malodor properties of oxidizing lozenges, as compared to breath mints and chewing gum. Healthy, young adult volunteers (N = 123; mean age 24.5 years) were measured for oral malodor-related parameters (whole mouth odor measured by 2 judges; tongue dorsum posterior odor using the spoon test; volatile sulphide levels; salivary levels of cadaverine and putrescine; and 2 versions of an oral rinse test) on the first afternoon of the study. They were then assigned randomly to one of 6 groups (2 brands of breath mints, chewing gum with no active ingredients, regular and full-strength oxidizing lozenges, and a no-treatment control), and instructed to employ the treatment before bedtime, the next morning, and in the early afternoon 3 hours prior to measurements, which were carried out 24 hours following baseline measurements. Volunteers also estimated the level of their own whole mouth and tongue odors at baseline and post-treatment. The data showed that, among treatments, only the full-strength oxidizing lozenge significantly reduced tongue dorsum malodor, as determined by the spoon test. The full-strength lozenge also yielded a significant increase in the modified oral rinse test, presumably due, at least in part, to residual oxidizing activity retained in the oral cavity. Self-estimations of whole mouth and tongue malodor by volunteers were significantly correlated with corresponding-judge assessments, suggesting some degree of objectivity in assessing one's own oral malodor. *J Periodontol 1997;68:1176–1181.*

Key Words: Halitosis/drug therapy.

Bad breath (halitosis, fetor ex ore) is a common complaint dating back to ancient times.[1,2] In most cases, breath odors originate within the oral cavity itself.[1–9] Bad breath is considered to result from production of sulphur-containing[3] and other gases,[4,5] by Gram-negative bacteria,[5–8] generally under anaerobic conditions.[5] In healthy persons, bad breath often derives from the posterior part of the tongue dorsum.[4,9] In many[6,8,10–15] but not all[7,16] studies, periodontal disease-related parameters have been found to be associated with bad breath levels. Other oral causes of bad breath include faulty restorations (e.g., overhanging restorations and leaking crowns) and sites of food impaction.[4]

Previous investigations have addressed the antimalodor properties of various mouth rinses, including those containing zinc compounds, essential oils, cetylpyridinium chloride, chlorhexidine, and 2-phase oil:water mouthwashes.[10,17,18] Few studies have addressed other oral products. In the present report, 123 young adults were tested for oral malodor parameters prior to and following sucking of various candies and lozenges, as well as chewing gum, as compared with a control group (no treatment). The data show that sucking of a full-strength lozenge with oxidizing properties reduces tongue dorsum malodor for 3 hours following use.

MATERIALS AND METHODS

The experiment consisted of a randomized clinical trial of 123 healthy volunteers (mean age 24.5 ± 3 years; 71 females) who were recruited by newspaper and university advertisements. Subjects who took antibiotics within 1 month prior to study, smokers, or those who had partial or complete dentures were excluded. The experiment was conducted according to an approved human subjects protocol and participants signed an informed consent form.

Subjects were split randomly into one of 6 groups: chewing gum (N = 22; chewing gum with no active ingredients;† breath mint 1‡ (N = 22); breath mint 2§ (N =

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†Warner Lambert, Morris Plains, NJ.
‡Tic Tac, Ferrero Frankfurt/M, Germany.
§O'dol Nice, Linger Fischer, Buhl, Germany.

Table 1. Experimental Protocol

Initial Measurement	Treatment	Treatment	Treatment	Final Measurement
4 p.m.–7 p.m.	bedtime	early morning	early afternoon	4 p.m.–7 p.m.
DAY 1		DAY 2		

19); oxidizing lozenge,‖ regular strength (N = 21); oxidizing lozenge,¶ full strength (N = 19); as well as a control group (N = 20; no treatment). Subjects were tested in random order, with researchers and odor judges blinded to group affiliation throughout.

The protocol of the experiment was similar to that previously used to compare mouth rinses.[18] Pre-treatment measurements were conducted between 4 p.m. and 7 p.m. of the afternoon of day 1. Subjects were instructed to reappear at the clinic at the same time on the next afternoon for post-treatment measurements. They were randomly assigned to 1 of the 6 groups. Those assigned to the lozenge/breath mint groups were asked to suck 1 lozenge/mint directly before bedtime on the same day (day 1); 1 in the early morning of the next day (day 2); and 1 in the early afternoon of day 2, 3 hours prior to their examination. Subjects in the chewing gum group were instructed to chew 1 piece of gum for 5 minutes directly before bedtime on the same day (day 1); 1 piece in the early morning of the next day (day 2); and 1 piece in the early afternoon of day 2, 3 hours prior to their examination. All subjects were instructed to continue their regular oral hygiene regimen, but to refrain from any oral hygiene activities, including toothbrushing, flossing, and rinsing, 3 hours before measurements. They were instructed to refrain from eating and drinking for at least 2 hours before measurements. The protocol of the experiment is summarized in Table 1.

Measurement Parameters

Volatile sulfur compounds (VSC). Determination of intraoral headspace VSC was carried out using a sulphide monitor.# Measurement was carried out essentially as previously reported,[8,10,15,19] using disposable straws, rather than teflon tubing. Participants were asked to refrain from talking for 5 minutes prior to measurement. The monitor was zeroed on ambient air, and measurement performed by inserting a disposable one-quarter inch plastic straw approximately 4 cm into the partially open oral cavity. Subjects were asked to breathe through their nose during measurement. Results were recorded as peak ppb sulphide equivalents.

‖Desaquick extra fresh, Roland Arzneimittel, Hamburg, Germany.
¶Desaquick forte, Roland Arzneimittel, Hamburg, Germany.
#Model 1170, Interscan Corp., Chatsworth, CA.

Oxygen depletion in expectorated milk rinse. Oxygen depletion in expectorated milk rinse was determined using the oratest oral rinse procedure,[20] as well as a modified version. Previous studies have shown that oratest scores are sensitive to changes in microbial counts and clinical parameters.[10,18,20,21] Briefly, volunteers rinsed vigorously for 30 seconds with 14 ml of sterile milk. Following expectoration, 3 ml of the sample was added to test tubes containing 0.12 ml of methylene blue solution (0.1%), and the time required for a blue-to-white color change over a 6 mm diameter at the bottom of the test tube was recorded. The oratest was performed directly following the judges' organoleptic measurements.

In the modified oratest, a 10 ml sample of the same milk expectorate was poured into a plastic disposable conic tube (12 cm height × 1.5 cm diameter)** and the extent (height in mm) of color change was recorded following 30 minutes' incubation at room temperature as above.

Organoleptic measurements. Organoleptic measurements of whole mouth malodor were performed by 2 judges, both with previous experience in assessing oral malodor (AG and EL). For judge scoring of whole mouth malodor, subjects were instructed to exhale briefly through the mouth, at a distance of approximately 10 cm from the nose of the 2 judges, sequentially. For self-assessment of whole mouth malodor, subjects were instructed to smell the odor emanating from their entire mouth by cupping their hands over mouth and nose, exhaling through the mouth and breathing in through the nose.[15] Odor measurements to assess the odor emanating from the posterior tongue dorsum were performed by one judge (EL), using a plastic spoon to scrape and scoop material from the far back region of the tongue dorsum.[4] Five seconds later, both the odor judge and subject assessed the spoon odor at a distance of approximately 5 cm. Results of all malodor assessments were independently rated in semi-integer intervals on a scale of 0 to 5[10] with description as follows: 0, no appreciable odor; 1, barely noticeable odor; 2, slight, but clearly noticeable odor; 3, moderate odor; 4, strong odor; 5, extremely foul odor. Judges and subjects were blinded from one another's scores throughout.

**Miniplast, Ein Shemer, Israel.

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Table 2. Mean Baseline Values for the Various Parameters

Parameter	Mean ± Standard Deviation
Whole mouth (Judge AG)*	1.95 ± 1.44
Whole mouth (Judge EL)*	1.20 ± 0.92
Whole mouth (Judges' mean)*	1.57 ± 0.98
Whole mouth (self-assessment)*	1.79 ± 1.07
Spoon test (Judge EL)*	1.95 ± 0.88
Spoon test (self-assessment)*	2.43 ± 1.19
Ln volatile sulphides†	4.26 ± 0.40
Ln oratest‡	4.93 ± 0.60
Modified oratest§	1.21 ± 1.29
Ln cadaverine‖	1.10 ± 1.49
Ln putrescine‖	2.14 ± 1.06

*On a semi-integer 0 to 5 scale.
†In ppb sulphide equivalents.
‡In minutes for color change on bottom of tube.
§Millimeters height color change after 30 minutes.
‖In peak ppm diamine.

Diamine analysis. Cadaverine and putrescine levels were determined in unstimulated whole saliva using high performance liquid chromatography (HPLC) as described previously.[10,22]

Statistical analysis. Comparisons between the various groups of the study were carried out using ANOVA, followed by Bonferroni's multiple comparisons. Several of the parameters (peak volatile sulphide levels, oratest, putrescine, cadaverine) were ln transformed in order to approximate normal distributions.

Comparisons between self-assessments and judges' scores were performed using paired *t*-tests. Associations between the various parameters were assessed by Pearson correlations. Multiple regression was applied in order to test the contribution of some of the post-treatment parameters (ln [cadaverine], ln [sulphide levels], and modified oratest) to the prediction of post-treatment mean judge score.

RESULTS

Mean baseline values of the various parameters for the entire subject group are summarized in Table 2. No differences in any of the parameters among the 6 treatment groups were detected at baseline ($P > 0.05$, ANOVA). Following treatment, significant differences among treatment groups were noted for 2 parameters: tongue dorsum posterior odor judge scores ($P = 0.025$, ANOVA) and modified oratest ($P = 0.018$, ANOVA).

The mean changes in each group following the various treatments are summarized in Table 3. Among the various treatments, only the full-strength oxidizing lozenge was found to significantly reduce tongue dorsum posterior malodor. This reduction was significant not only with respect to the no-treatment control ($P = 0.009$), but also with respect to the chewing gum group ($P = 0.002$), breath mints 1 and 2 ($P = 0.023$ and $P = 0.016$, respectively), and the regular-strength oxidizing lozenge group ($P = 0.003$).

The full-strength oxidizing lozenge also yielded the largest improvements (decreases) in self-assessment whole mouth and self-assessment tongue posterior odor scores, as well as whole mouth odor scores (judge EL), but these did not reach significant levels.

With respect to the modified oratest, use of the full-strength oxidizing lozenge was found to be associated with significant increases in the extent of oxygen consumption in the rinsed milk samples. This increase was significant not only with respect to the no-treatment control ($P = 0.0025$), but also with respect to the chewing

Table 3. Changes From Baseline Means

Tested Parameter	Chewing Gum	Breath Mint 1	Breath Mint 2	Regular Oxidizing Lozenge	Full-Strength Oxidizing Lozenge	Control (No Treatment)
Whole mouth (Judge AG)*	−0.54 ± 1.28	−0.54 ± 1.17	−1.47 ± 1.58	−1.40 ± 1.75	−1.29 ± 1.32	−1.00 ± 1.60
Whole mouth (Judge EL)*	0.02 ± 1.25	−0.07 ± 0.76	−0.08 ± 1.31	0.02 ± 0.77	−0.29 ± 0.89	−0.10 ± 0.84
Whole mouth (Judges' mean)*	−0.26 ± 0.95	−0.31 ± 0.78	−0.78 ± 1.17	−0.69 ± 1.03	−0.79 ± 0.77	−0.55 ± 0.84
Whole mouth (self-assessment)*	0.14 ± 0.77	−0.09 ± 1.11	−0.21 ± 1.18	−0.17 ± 1.06	−0.36 ± 1.13	0.27 ± 0.79
Spoon test (Judge EL)*	0.20 ± 0.93	−0.07 ± 0.82	0.00 ± 0.88	0.14 ± 0.74	−0.74 ± 0.99¶	0.05 ± 1.15
Spoon test (self-assessment)*	0.02 ± 1.12	−0.29 ± 1.23	−0.39 ± 1.65	0.10 ± 1.294	−0.82 ± 0.99	−0.12 ± 0.65
Ln volatile sulphides†	0.003 ± 0.25	−0.02 ± 0.35	−0.09 ± 0.34	0.03 ± 0.41	−0.09 ± 0.23	−0.06 ± 0.38
Ln oratest‡	−0.03 ± 0.53	−0.03 ± 0.69	0.16 ± 0.58	−0.20 ± 0.68	−0.20 ± 0.61	0.05 ± 0.59
Modified oratest§	−0.14 ± 1.16	−0.23 ± 1.35	−0.74 ± 1.49	0.09 ± 1.65	0.82 ± 1.85#	−0.62 ± 1.16
Ln cadaverine‖	0.06 ± 1.02	−0.42 ± 0.85	−0.16 ± 0.65	−0.53 ± 0.86	0.31 ± 1.34	−0.34 ± 0.70
Ln putrescine‖	−0.19 ± 0.75	−0.28 ± 1.04	−0.45 ± 0.67	−0.29 ± 0.73	−0.39 ± 0.78	−0.32 ± 0.64

*On a semi-integer 0 to 5 scale.
†In ppb sulphide equivalents.
‡In minutes for color change on bottom of tube.
§Millimeters height color change after 30 minutes.
‖In peak ppm diamins.
¶Significant difference among groups, $P = 0.0254$ (ANOVA); full-strength oxidizing lozenge scores differed significantly from 1) chewing gum group, $P = 0.0015$; 2) breath mint 1 group, $P = 0.0228$; 3) breath mint 2 group, $P = 0.0156$; 4) regular oxidizing lozenge, $P = 0.0033$; and 5) no treatment control group, $P = 0.0091$.
#Significant difference among groups, $P = 0.0184$ (ANOVA); full-strength oxidizing lozenge scores differed significantly from 1) chewing gum group, $P = 0.0389$; 2) breath mint 1 group, $P = 0.024$; 3) breath mint 2 group, $P = 0.0013$; and 4) no treatment control group, $P = 0.0025$.

gum group ($P = 0.0389$), as well as breath mints 1 and 2 ($P = 0.024$ and $P = 0.0013$, respectively). Modified oratest levels following use of regular versus full-strength oxidizing lozenges were not significantly different from one another. Similar trends to those found for the modified oratest were observed with the original oratest procedure, but differences among groups were not significant.

The increase in the modified oratest scores following use of the full-strength oxidizing lozenges appears anomalous, since it would ostensibly indicate an increase, rather than decrease, in microbial activity.[10,18,20,21] However, since the efficacy of the lozenges is based on oxidizing activity, we guessed that the observed increase was due, at least in part, to the depletion of oxygen brought about by active components of the lozenge. Indeed, in vitro experiments in which lozenges were mixed with sterile saliva and added to sterile milk containing methylene blue yielded rapid color change indicative of oxygen depletion, whereas control tubes containing sterile saliva and milk alone did not yield color change (data not shown). Future research is necessary to determine whether the oxidizing agents of the lozenge indeed show retentivity on the tongue surface for 3 hours following use.

Pearson correlation coefficients were used to compare associations among the various parameters. At baseline and following treatment, both judge's scores of whole mouth malodor were moderately associated with one another ($r = 0.35$, $P < 0.001$). Both judges' initial whole mouth malodor scores were related to volatile sulphide levels, yielding r values of 0.27 ($P = 0.003$) and 0.39 ($P < 0.001$) for judges AG and EL, respectively. However, neither of the initial odor judge scores was significantly related to cadaverine levels ($P > 0.05$). Following treatment, correlations between odor judge scores and volatile sulphide levels were significant for judge AG ($r = 0.27$, $P = 0.003$), but not for judge EL ($r = 0.16$, $P = 0.072$). Conversely, EL post-treatment scores were highly associated with cadaverine ($r = 0.31$, $P = 0.001$), whereas AG post-treatment whole mouth scores were not ($r = 0.16$, $P = 0.09$). Multiple regression analysis of post-treatment results showed that both cadaverine and volatile sulphides factored equally in accounting for mean whole mouth odor judge scores ($P = 0.01$ for both parameters), yielding a multiple r of 0.36 ($P < 0.001$). In contrast, only volatile sulphide scores factored into the regression equation accounting for baseline mean odor judge scores, yielding a multiple r of 0.39 ($P < 0.001$).

Self-assessments of whole mouth malodor were not significantly different from the corresponding mean judge scores at baseline ($P = 0.073$, paired t-test), but were significantly higher than corresponding mean judge scores post-treatment ($P < 0.001$, paired t-test). Self-assessment of spoon scores (indicative of odor of the tongue dorsum posterior) was higher than corresponding judge scores both prior to, and following treatment ($P < 0.001$ and $P = 0.006$, respectively; paired t-test). Self-assessments of whole mouth, pre- versus post-treatment, were highly associated with one another ($r = 0.52$, $P < 0.001$), as were self scores of the spoon odor prior to versus following treatment ($r = 0.49$, $P < 0.0001$). Corresponding pre- versus post-treatment odor judge scores were less strongly associated with one another ($r = 0.33$, $P < 0.001$; and $r = 0.35$, $P < 0.001$ for whole mouth scores of AG and EL, respectively; and $r = 0.40$, $P < 0.001$ for spoon scores of judge EL).

Interestingly, self-assessments of whole mouth were significantly associated with corresponding mean judge scores, both at baseline ($r = 0.20$, $P = 0.024$) and post-treatment ($r = 0.23$, $P = 0.009$). Furthermore, self-scoring of spoon odor versus corresponding judge (EL) scores yielded highly significant correlations of $r = 0.25$, $P = 0.005$ (baseline) and $r = 0.35$, $P < 0.001$ (post-tretament), respectively. Self spoon scores were also significantly correlated with volatile sulphide scores, both pre- and post-treatment ($r = 0.20$, $P = 0.03$; and $r = 0.25$, $P = 0.005$, respectively), but not with levels of salivary cadaverine ($P > 0.05$).

Salivary cadaverine concentrations were marginally associated with sulphide levels both before and following treatment ($r = 0.20$, $P = 0.03$; and $r = 0.19$, $P = 0.043$, respectively). In contrast, putrescine levels were not related to sulphide levels ($P > 0.2$).

Correlations between the modified oratest and the previous oratest technique were high, both at baseline and following treatment ($r = -0.61$, $P < 0.001$; and $r = -0.68$, $P < 0.001$, respectively).

DISCUSSION

Although mouth rinses have been tested for their effectiveness in reducing oral malodor parameters in a variety of studies,[10,17,18] few investigations have been conducted using other kinds of oral products. In the present study, we compared the efficacy of breath mints, oxidizing lozenges, and chewing gum in reducing oral malodor over a 3-hour period.

Among the various treatments, only the full-strength oxidizing lozenge was effective in reducing tongue malodor, as determined by the spoon test. Furthermore, this decrease was significant, as compared to all other treatment groups (P values ranging from 0.025 to 0.009). Although the full-strength oxidizing lozenge also yielded the largest improvements (decreases) in other parameters; i.e., self-assessment scores (whole mouth and tongue dorsum posterior), and whole mouth odor scores (judge EL), these did not reach significant levels. To our knowledge, this is the first report demonstrating a 3-hour anti-malodor effect of any lozenge. More recent findings (unpublished) have demonstrated a 90-minute effect for the regular-strength oxidizing lozenge in reducing posterior tongue malodor.

The anti-malodor effect may be due to the activity of dehydroascorbic acid, which is generated by peroxide-mediated oxidation of ascorbate present in the lozenges. Indeed, in vitro malodor reduction by mixtures containing oxidizing agents and ascorbic acid has been previously demonstrated.[22]

Although gum chewing per se is considered to be helpful in reducing bad breath during the day by stimulating saliva flow and mechanical cleansing,[4] the chewing gum employed in this study, devoid of active ingredients, was not effective in reducing malodor parameters 3 hours following use.

Gentle scraping of the back of the tongue with a plastic spoon, as performed here, has been used successfully for several years in our clinical assessment of the source of oral malodor.[4] We have recently begun to employ this test as a measurement parameter in research protocols.[10] In addition to its ability to distinguish anti-malodor activity among the groups, the "spoon test" appears to have potential in self-assessment. Subjects' scores of the spoon malodor, both at baseline and post-treatment, correlated significantly with corresponding odor judge scores, as well as with volatile sulphide measurements.

As in previous studies, odor judge scores were significantly associated with sulphide monitor results, although the correlation coefficients obtained were not as high as those found elsewhere.[1] Furthermore, changes following treatment were slight and not significant. Similarly, although cadaverine was marginally related to volatile sulphide scores, and factored significantly (together with sulphide levels) in explaining post-treatment odor judge scores, the relationships between this diamine and other malodor-associated parameters were weaker than those found in a previous investigation.[23] One reason for the lower degree of association may be the relatively young age and good health of the subjects studied here.

This is the first study to employ the modified oratest. Its main advantage is that the color change is not followed continuously, but rather at a fixed interval (30 minutes). The modified oratest was highly associated with the previous oratest technique, and was more sensitive to differences among groups than the previous test. Although the oratest was originally devised as a test of oxygen consumption by the expectorated oral microbiota, the results presented here suggest the potential of the modified oratest in monitoring substantivity of oxidizing agents in the oral cavity.

Correlations between the 2 judges, although significant, were moderate, even though both judges had previously participated in clinical malodor studies. In a previous study, we compared inter-examiner scores among 7 judges and found that the Pearson correlation coefficients ranged from 0.14 ($P = 0.135$) to 0.49 ($P < 0.001$).[24] One potential factor in accounting for inter-examiner variability is that each judge exhibits a different sensitivity towards different "notes" of oral malodor. The post-treatment odor judge scores presented here provide initial support for such a hypothesis. Whereas judge EL's post-treatment scores were highly associated with cadaverine ($P = 0.001$) but not volatile sulphides ($P = 0.072$), judge AG's post-treatment scores were associated with volatile sulphides ($P = 0.003$), but not with cadaverine ($P = 0.087$). When the molecular compositions of the various kinds of oral malodor become better characterized, it should be possible to test this hypothesis more thoroughly.

In a recent study, we addressed the question of whether people can objectively gauge their own bad breath, in a group of 52 subjects, most of whom had a complaint of bad breath. Subjects were asked to rate the odor emanating from their mouths in the same manner as described here (i.e., by cupping hands over mouth). In the previous study, no correlation was found between self-estimation of whole mouth malodor and objective measurements. However, in the present investigation, significant associations were found comparing self-scoring of malodor with odor judge scores, as well as with volatile sulphide levels. One possible explanation for this discrepancy is that the group of subjects in the previous study were concerned about having bad breath and exhibited elevated psychopathological scores as compared with a normal population.[25] In contrast, the subjects studied here were healthy young adults who had no specific complaint regarding bad breath and were recruited based on financial remuneration. As with whole mouth odor, self-estimation of spoon scores was also significantly associated with odor judge scores and sulphide levels, both pre- and post-treatment. These data raise the possibility that in the general population, some degree of objective self-evaluation regarding oral odor is possible. This objectivity, however, may still be mitigated by personal sensitivity to one's own odor. As found in the previous investigations,[24,25] subjects in the present study tended to score their own tongue odor more severely than the corresponding odor judge scores, both prior to and following treatment. Moreover, subjects' baseline and post-treatment self-scores were more highly associated with one another than corresponding odor judge scores.

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BDA Fact File

Bad breath

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Bad breath (halitosis) is a common problem which often comes from the activity of bacteria in the mouth. Although there is no way of knowing for sure, most adults probably suffer from bad breath occasionally, with perhaps a quarter suffering on a regular basis. This fact file looks at the causes and at what dentists can do to help.

Is bad breath always treatable?
In the past, bad breath was often considered to be an incurable affliction. However, in recent years it has become increasingly evident that bad breath is usually treatable once a proper diagnosis is made.

The main problem is knowing whether we have it or not, because we are poor judges of our own breath odour. Some people suffer from bad breath without knowing it, while others build up exaggerated fears about breath odour even though they do not have it. The best way to find out whether we have bad breath is to ask for someone else's opinion. If we don't ask, other people are unlikely to tell us. And since bad breath can sometimes - fortunately rarely - be a sign of a significant general health problem, we should not be reluctant to tell people dear to us that they have a bad breath problem.

What should I do if I have bad breath?
If you have reason to believe that there is a problem, then see your dentist first, since bad breath often comes from the mouth itself.

When you see the dentist, it is a good idea to explain in advance that you will be asking for advice about bad breath. Also, try to go with someone who is familiar with the problem, (a family member or close friend, for example), to help give the dentist an objective picture of how bad the odour really is, how long it has been going on, and when it improves or gets worse. Since bad breath often varies, the person accompanying you can also help determine whether the odour at the time of the appointment resembles, both in character and intensity, the odour that is generally troublesome.

If the dentist knows that the consultation is about bad breath, you may be asked not to eat, drink, smoke, chew gum, suck confectionery, use mouthwashes, breath fresheners etc., so that the odour will be more typical. You should also avoid using perfumed cosmetic products, such as perfume, aftershave and scented lipstick prior to the appointment, since it can interfere with the odour assessment. If the dentist is not told about the reason for the consultation beforehand, do these things anyway and tell the dentist that you have prepared for the appointment in this way.

What will happen at my appointment?
Your dentist will ask questions to help determine the possible causes of the odour, and then compare the odour coming from your mouth and nose. In most cases (about 85-95%) the odour comes from the mouth rather than the nose. This is an indication that bacterial activity somewhere in the mouth is responsible. If the odour comes mostly from the nose, then the nasal passages may be involved.

Your dentist may also make measurements using a sulphide monitor to help in diagnosis and treatment, since volatile sulphur compounds are often associated with bad breath.

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This table summarises different odour-related problems, and their possible causes.

Problem	Possible cause or source of malodour
Odour after fasting, dieting, sleeping, taking medications, prolonged speaking, exercise	dryness in the mouth, insufficient saliva flow
Gums bleed and/or smell	gum problems, poor cleaning between teeth
Odour upon talking	postnasal drip on back of tongue
Odour at onset of menstrual cycle	swelling of gums
Small whitish stones with foul odour appear on Tongue, for example following coughing	tonsoliths from crypts in tonsils
Odour appears suddenly from mouth of young children	onset of throat infection
Odour appears suddenly from nose of young children	foreign body placed in nose
Odour appears suddenly from entire body of young children	foreign body placed in nose
Taste or smell of rotten fish	trimethylaminuria (rare)
Odour in denture wearers	dentures kept in mouth at night or not cleaned properly
Odour from nose	sinusitis, polyps, dryness, foreign body, hindered air or mucus flow
Bad taste all day long	poor oral hygiene, gum disease, excessive bacterial activity on tongue

Where does the odour come from?

Most cases of bad breath appear to be due to the breakdown of proteins by a variety of micro-organisms. Several of the breakdown products are foul smelling gases.

In people with healthy teeth and gums, the odour usually comes from the far back region of the tongue, and grows stronger when the patient starts talking. The dentist can sample this area using a plastic spoon. The odour coming from the spoon sample may then be compared to the overall odour.

Although we do not know why, the very back of the tongue is an important source of bad breath, possibly as a result of postnasal drip, which can get stuck on the tongue and is then broken down by bacteria on the tongue surface.

If the back of the tongue is the problem, then the dentist can recommend a method of cleaning the area with a specially designed tongue scraper (in some countries, tongue cleaning is a common and ancient practice). Several types of tongue cleaners are now

available in the UK. It takes time and patience to overcome the gagging reflex, but eventually, tongue cleaning becomes easy. Care should be taken to clean the back of the tongue thoroughly yet gently, without inflicting pain or sores.

Can gum disease cause bad breath?
In some people, bad breath is associated with gum disease, especially if rubbing the areas between the teeth and gums yields a foul odour. Your dentist can help prevent and treat gum diseases in various ways, depending on the type and extent of the problem, but your own daily home care makes all the difference in the world in maintaining gum health between appointments. Cleaning of the spaces between the teeth is of great importance. One home tip to healthy gums (and less bad breath) is to smell the odour coming from the dental floss following each passage, and to work to clean those areas more carefully. People with gum disease often have higher levels of odour coming from their tongue, as well. In certain instances your dentist may recommend special toothpicks to clean between your teeth. You can also smell the odour on the toothpicks to find out where the odour is coming from.

What type of treatment is there?
Your dentist may recommend dental treatment, if there are other areas in which bacteria and food can become trapped and cause odour. The dentist may also suggest daily rinsing with one of several available mouthwashes which have been scientifically shown to reduce breath odours[1,2]. Recent mouthwash developments include a two-phase mouthwash now available in the UK, that visibly traps and removes bacteria and debris, and several mouthwashes with a variety of antibacterial anti-odour properties. In coming years, other types of oral products specifically geared to treat bad breath (for example, special tablets) will probably be introduced as well.

Your dentist may also refer you to clinics that specialise in identifying breath odours, or to other medical experts. Since, in some instances, patients get overly concerned about this problem (especially since it is so difficult to assess one's own breath), the dentist may also recommend that you discuss this problem over with a psychological professional, to help you bring it into the proper perspective.

What can I do?
In all probability, professional diagnosis and treatment can help eliminate bad breath. However, it is sometimes difficult for us to sense the improvement ourselves. In this case, a family member or close friend can also provide important feedback and reinforcement.

Listed below are some of the Do's and Don'ts regarding bad breath. Remember, bad breath is a problem that needs professional attention. Don't mask it - deal with it.

Do's
- Visit your dentist regularly.
- Have your teeth cleaned periodically by a dental professional.
- Floss or otherwise clean between your teeth, as recommended by your dentist. Choose unscented floss so that you can detect those areas between your teeth that give off odours, and clean them more carefully.
- Brush your teeth and gums properly.
- Ask your dentist to recommend a tongue cleaner. Clean your tongue all the way back gently, but thoroughly.
- Drink plenty of liquids, but not too much coffee.
- Chew sugar-free gum, especially if your mouth feels dry. Chewing parsley, mint, cloves or fennel seeds may also help.
- Clean your mouth after eating or drinking milk products, fish and meat.
- Unless your dentist advises otherwise, soak dentures overnight in antiseptic solution to prevent odour build-up.
- Get control over the problem. Ask a family member to tell you whenever you have bad breath.
- If someone in your family or a close friend has bad breath, find a kind way to let them

know. If you can't tell them directly, leave this fact file lying around. They may get the message.
- Ask your dentist to recommend a mouthwash which has been shown to be clinically effective in fighting bad breath. Use it most effectively right before sleeping.
- Eat fresh, fibrous vegetables such as carrots.

Don'ts
- Don't let your concern about having bad breath run your life. Don't be passive.
- Don't be depressed. Get help. Don't ignore your gums - you can lose your teeth as well as smell bad.
- Don't drink too much coffee - it may make the situation worse.
- Don't forget to clean behind the back teeth in each row.
- Don't brush your tongue with a regular toothpaste - it's better to dip your toothbrush in mouthwash for tongue cleaning.
- Don't run to the gastroenterologist for concerns of having bad breath - it usually comes from the mouth and almost never from the stomach.
- Don't give mouthwash to very young children, as they can swallow it.
- Don't clean your tongue so hard that it hurts.
- Don't rely on mouthwash alone - practise complete oral hygiene.

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Halitosis in children

Erica Amir, DMD, Rosa Shimonov, DMD, and Mel Rosenberg, PhD

Objective: To determine the relationship between oral parameters and halitosis in children whose parents complained of malodorous breath.

Methods: Twenty-four children (ages 5 to 14) were examined at 3 appointments. After the second appointment oral hygiene instructions were given. Malodor-related parameters included odor judge scores (whole mouth, tongue, nose, and interdental areas), sulfide levels, and microbiologic tests (Oratest and BANA). Dental-related parameters included plaque index, dental index (DMFT), food impaction, bleeding, and tongue coating. Statistical analyses included analysis of variance, paired *t* tests, Pearson correlations, and multiple regression.

Results: Whole mouth odor was significantly associated with plaque index levels ($r = 0.64$, $P = .001$) and Oratest ($r = -0.57$, $P = .005$). Whole mouth malodor was significantly associated with tongue dorsum posterior odor ($r = 0.641$, $P = .001$) and was higher in subjects with interdental odor ($P = .003$). Tongue odor was also significantly associated with nasal malodor ($r = 0.57$; $P = .004$). Sulfide levels were correlated with oral malodor levels only at the second appointment ($r = 0.46$, $P = .02$).

Conclusions: The data suggest that, as in adults, oral malodor in children is related primarily to oral factors. Correlations between nasal and oral malodor were evident, suggesting that postnasal drip plays a major role.
(J Pediatr 1999;134:338-43)

Bad breath (halitosis) is a common complaint in the adult population.[1] In most cases it results from microbial activity on the dorsal tongue and between the teeth and periodontium.[2-6] Outside the oral cavity bad breath has been associated with chronic sinusitis,[7,8] upper and lower respiratory tract conditions, various systemic diseases, and use of certain drugs.[9] Transient foul breath is also commonly observed after tonsillectomy.[10,11] Bad breath as the result of gastrointestinal disorders is considered to be extremely rare.[12]

Bad breath is associated with volatile sulfur compounds such as hydrogen sulfide, methyl mercaptan, dimethylsulfide, and others.[1-3,13-15] Other components such as cadaverine,[16] indole, and skatole may also be involved.[3] Methods of measurement of bad breath and related parameters include organoleptic examination by odor judges, chromatographic analyses, sulfide monitors, and color tests.[17]

Although clinical experience suggests that bad breath occurs in children and adults, the few studies that have addressed this phenomenon have dealt with populations of children with upper respiratory tract infections.[7,8,11] The purpose of this investigation was to compare bad breath parameters and dental indexes in a group of children with a primary family report of bad breath.

METHODS

Patients

The study included 24 healthy children (13 girls) ranging in age from 5 to 14 years whose parents complained about their bad breath. Potential participants were made aware of the study in a local radio interview. None of the children in the study wore orthodontic appliances or retainers.

Study Protocol

The examinations were performed in the morning at 3 separate appointments at intervals of 6 to 9 days. The children were asked to refrain from toothbrushing, eating, drinking, or chewing gum for at least 2 hours before the examination. Subjects participated in the clinical studies after their parents provided informed consent to a protocol that was reviewed and approved by the appropriate institutional review board.

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At the first appointment a questionnaire was given to the accompanying parent and to the child: it included personal data, medical and dental health history, and pertinent oral habits (eg, frequency of toothbrushing, use of mouthrinses, mouth breathing). Bad breath and dental-associated parameters were subsequently measured. At the second appointment bad breath parameters were measured, and then oral hygiene instructions were given to the children in the presence of the accompanying parent. At the third and final appointment the bad breath parameters were again assessed. Parents were asked whether they noticed any improvement in the bad breath of the children. Bad breath and dental-related parameters were measured as described in the following text.

Table I. Mean (± SD) scores of malodor-related parameters at the 3 appointments

Parameter	First appointment	Second appointment	Third appointment
Whole mouth	1.9 ± 0.8	1.6 ± 0.6*	1.6 ± 0.7*
Count-to-20	2.6 ± 2.8	2.0 ± 1.3*	1.7 ± 1.8*
Nose	0.6 ± 0.7	0.3 ± 0.6†	0.3 ± 0.6*
Tongue anterior	2.2 ± 1.0	1.6 ± 0.9†	1.3 ± 0.8†‡
Tongue posterior	2.0 ± 1.0	1.6 ± 0.9*	1.7 ± 0.6*‡
Peak sulfides	4.3 ± 0.4	4.2 ± 0.4	4.2 ± 0.5
Oratest	3.3 ± 0.8	3.5 ± 0.5	3.4 ± 0.7

*Significantly different from first appointment ($P < .05$).
†Significantly different from first appointment ($P < .005$).
‡Significantly different from second appointment ($P < .05$).

Organoleptic Measurements

Organoleptic measurements were carried out throughout the study by a single experienced examiner whose scores have been compared with those of other judges and measurement techniques in previous studies.[18,19] Six parameters were used: (1) whole mouth breath,[14,15] (2) breath odor while speaking ("count-to-20 test"),[20] (3) tongue dorsum anterior ("wrist lick test")[19]; (4) tongue dorsum posterior ("spoon test"),[12,21] (5) nasal odor,[20] and (6) floss malodor from interdental areas.[14] (1) For judge scoring of whole mouth breath, subjects were instructed to exhale briefly through the mouth at a distance of approximately 10 cm from the nose of the judge. (2) For measurement of breath odor while speaking, children were asked to count until 20, and the first number at which bad breath was detected was recorded. (3) Odor judge measurements to assess the odor emanating from the anterior tongue dorsum were performed by asking the child to lick the wrist with tongue extended. Five seconds later the odor judge assessed the odor on the wrist at a distance of approximately 5 cm. (4) Odor judge measurements to assess the odor emanating from the posterior tongue dorsum were performed with a plastic spoon by scraping and scooping material from the very back region of the tongue dorsum. Five seconds later the odor judge assessed the odor on the spoon at a distance of approximately 5 cm. (5) For judge scoring of nasal breath, subjects were instructed to exhale briefly through the nose at a distance of approximately 10 cm from the nose of the judge. Results of the previously mentioned malodor assessments were scored in semi-integer intervals on a scale of 0 to 5 with description as follows: 0, no appreciable odor; 1, barely noticeable odor; 2, slight but clearly noticeable odor; 3, moderate odor; 4, strong odor; 5, extremely foul odor. To determine the presence or absence of interdental odor, floss (unwaxed) was passed between the posterior teeth and scored as positive or negative by the judge at a distance of approximately 5 cm.

Volatile Sulfur Compounds

The determination of intraoral headspace volatile sulfur compounds was carried out with a sulfide monitor (model 1170; Interscan Corp, Chatsworth, Calif). Measurement was carried out essentially as previously reported[14,15] with disposable straws rather than Teflon tubing. Participants were asked to refrain from talking for 5 minutes before the measurement was taken. The monitor was zeroed on ambient air, and the measurement was performed by inserting a disposable ¼-inch plastic straw approximately 4 cm into the partially open oral cavity. Subjects were asked to breathe through their nose during measurement. Results were recorded as peak ppb sulfide equivalents.

Oratest (Oxygen Depletion in Expectorated Milk Rinse)

Oxygen depletion in expectorated milk rinse was determined with the Oratest oral rinse procedure. Previous studies have shown that Oratest scores are sensitive to changes in microbial counts and are associated with clinical parameters.[22,23] Children rinsed vigorously for 30 seconds with 10 mL sterile milk. After expectoration, 3 mL of the sample was added to test tubes containing 0.12 mL methylene blue solution (0.1%), and the time required for a blue-to-white color change over a 6-mm diameter at the bottom of the test tube was recorded. The Oratest was performed directly after the judge's organoleptic measurements.

BANA Assay

The BANA reagent strip (Perioscan, Oral-B Laboratories, Redwood City, Calif)[24] was used according to manufacturer's instructions. Plaque samples were removed with a dental explorer and applied directly to the strip. Tongue samples were obtained by scraping the posterior tongue dorsum

Table II. Correlations with whole mouth malodor at initial appointment

Parameter	Pearson correlation coefficient (r =)	Significance (P =)
Plaque index	0.64	0.001
Nasal odor	0.42	0.04
Tongue dorsum anterior odor	0.50	0.01
Tongue dorsum posterior odor	0.64	0.001
Presence of interdental odor	NA	0.003
Oratest	−0.57	0.003
No. of bleeding sites	0.51	0.01

NA, Not applicable (t test used).

Table III. Correlations with tongue dorsum posterior malodor at initial appointment

Parameter	Pearson correlation coefficient (r =)	Significance (P =)
Whole mouth odor	0.64	0.001
Nasal odor	0.57	0.004
Tongue dorsum anterior odor	0.44	0.03
Presence of interdental odor	NA	0.03
Mouth breather	NA	0.01
Brushes regularly	NA	0.001
Presence of tongue coating	NA	0.03
Bleeding on brushing	NA	0.03
Food impaction present	NA	0.001

NA, Not applicable (t test used).

with a disposable plastic spoon and were applied directly onto the reagent card. All samples were applied at the bottom of the card. Saliva samples were taken before the dental examination by asking the children to spit into a petri dish. Aliquots (approximately 10 μL) of saliva were immediately applied onto the reagent card. The reaction was immediately initiated by application of water to the upper strip containing the fast black dye, and then both strips were brought together when the testing card was folded. A clip was positioned to ensure the proximity of both strips, and the card was then placed in an incubator at 55° C for 15 minutes. Results were recorded as either strong, dark blue spots (score = 2), weak, light blue spots (score = 1), or no color change (score = 0).

Dental Parameters

Dental parameter measurements were carried out by a single experienced examiner at the first appointment (EA) who was blinded to the bad breath measurements results. The following parameters were checked: (1) DMFT (total number of decayed [D], missing/extracted [M] due to decay, and filled [F] teeth [T]) of the deciduous and permanent dentition, (2) total number of teeth with advanced decay, with extreme mobility, or in the process of exfoliation or eruption, (3) presence of food impaction, (4) presence of tongue coating, (5) plaque index according to Silness and Loe,[25] and (6) number of bleeding sites on probing.

Statistical Analysis

Several of the parameters (peak volatile sulfide levels; Oratest) were transformed logarithmically to approximate normal distributions. Comparison between individual parameters at the various appointments were carried out with analysis of variance with Bonferroni's multiple comparison correction.

Comparisons between self-assessments and judges' scores were performed with paired t tests. Associations between the various parameters were assessed by Pearson correlations. Multiple regression was performed to test the contribution of the plaque index and Oratest scores to the prediction of initial whole mouth malodor levels.

RESULTS

The mean levels (±SD) of the various malodor-related parameters at the 3 appointments are presented in Table I. At the first appointment dental parameters were also evaluated. Mean levels (±SD) of plaque index and DMFT were 1.2 ± 0.5 and 5.0 ± 3.0, respectively. Mean numbers (±SD) of mobile teeth, exfoliating teeth, erupting teeth, teeth with advanced decay, and bleeding sites were 1.4 ± 1.6, 0.2 ± 0.5, 1.5 ± 1.9, 0.2 ± 0.4, and 0.8 ± 2.1, respectively.

As shown in Table II, at the first appointment significant correlations were observed between whole mouth malodor levels and other odor assessments such as nasal odor, tongue dorsum anterior odor, and tongue dorsum posterior odor. Whole mouth malodor was also significantly associated with other parameters such as plaque index, the Oratest, and the number of bleeding sites.

The "count-to-20" test (odor on speaking) was highly associated with the presence of tongue coating (P = .0007) and food impaction (P = .0009), with more moderate correlations with mouth breathing (P = .01) and presence of bleeding on brushing (P = .055).

As summarized in Table III, tongue dorsum posterior odor was significantly correlated with a wide range of odor and dental parameters: whole mouth odor, nasal odor, tongue dorsum anterior odor, interdental (floss) odor, mouth breathing, regular brushing habits, presence of tongue coating, presence of bleeding on toothbrushing, and presence of food impaction.

Tongue dorsum anterior odor appeared to be highly related ($P = .0002$) to a history of throat infection and was also associated with regular brushing habits ($P = .001$), use of mouthwash ($P = .003$), and bleeding on brushing ($P = .001$).

Plaque index levels were associated with brushing habits ($P = .001$), bleeding on brushing ($P = .012$), tongue coating ($P = .019$), food impaction ($P = .018$), floss odor ($P = .02$), mouth breathing ($P = .02$), and history of throat infections ($P = .02$).

Correlations were found comparing Oratest results with both whole mouth ($r = -0.572$, $P = .003$), tongue dorsum posterior ($r = -0.53$, $P = .008$) odor levels, DMFT ($r = 0.42$, $P = .04$), and the presence of interdental odor ($P = .017$). The negative correlations with the Oratest were as expected, because the shorter the time required for color change was, the higher the microbial load was in the sample.

At the first appointment peak volatile sulfide levels were associated with the number of bleeding sites ($r = 0.46$, $P = .03$) but not with malodor levels. BANA test scores were not associated with malodor parameters. This result may be related to the observation that clear-cut positive scores were detected in only 4 (5.5%) of the plaque samples and none of the saliva or tongue samples tested.

At the second appointment whole mouth malodor was significantly associated with peak volatile sulfide levels ($r = 0.46$, $P = .02$). Nasal odor was correlated with tongue dorsum posterior odor ($r = 0.48$, $P = .02$). At the third and final appointment, whole mouth malodor was highly associated with tongue dorsum posterior odor ($r = 0.80$, $P < .001$) and was also related to tongue dorsum anterior odor ($r = 0.46$, $P = .02$). Peak volatile sulfide levels were significantly associated with nasal malodor ($r = 0.54$, $P = .006$).

In a comparison of the individual malodor-related parameters at all 3 appointments, only in the case of the Oratest were results of all 3 visits significantly associated with one another ($P < .003$).

Because both plaque index and Oratest correlated with whole mouth malodor but not with one another, stepwise forward linear multiple regression analysis was carried out to test the contribution of the plaque index and Oratest scores in the prediction of initial whole mouth malodor levels. A multiple r of 0.78 was obtained, with both plaque index and Oratest contributing significantly ($P < .001$ and $P = .005$, respectively) to the regression equation.

No significant differences were found between male and female children.

DISCUSSION

To our knowledge this is the first study to examine the potential relationship between oral health parameters and bad breath in a group of children. This investigation suggests that bad breath in children is generally similar to that found in adults. The types of odors encountered (whole mouth odor, count-to-20 test, odor from the front and back of the tongue dorsum, interdental odor, nasal odor) were highly reminiscent of analogous odors in adults. This perhaps is less surprising when one considers that putative periodontopathogens, considered important in the formation of oral malodor in adults,[3] are present in the dental plaque of most children.[26] Furthermore significant positive correlations were found in this study between bad breath parameters and dental parameters such as plaque index, number of bleeding sites, food impaction, tongue coating, and regular toothbrushing.

In general, although bad breath was the parents' complaint, initial malodor scores were not particularly high (eg, mean initial whole mouth score of 1.9), suggesting that the parents entering the study were concerned about their children having even slightly bad breath. It is interesting that significant reductions in malodor scores were already apparent at the beginning of the second appointment, even though oral hygiene instructions were given only at the end of the second appointment. This result may be attributed to heightened awareness of the potential link between malodor and oral hygiene because the study was carried out at a dental clinic, and (2) oral measurements were carried out during appointment 1. In 65% of the cases an improvement in bad breath after the 3 appointments was also noted by the parents or the children themselves.

In addition to the involvement of the oral cavity per se, the study underlies the importance of nasal odor in this group and suggests a potential relationship between nasal odor and oral parameters. Nasal odor was related to several specific oral odors, particularly with tongue dorsum posterior malodor ($r = 0.57$, $P = .004$), the presence of tongue coating ($P = .006$), and brushing habits. It has previously been postulated[12,20] that oral malodor in many adults is related to tongue odor caused by putrefaction of postnasal drip, which accumulates on the back of the tongue. The results presented here suggest that this hypothesis holds for children as well. At the first and third appointments, whole mouth malodor and posterior tongue dorsum odor were strongly associated with one another ($r = 0.64$ and 0.80, respectively; $P < .001$). In this context it is noteworthy that in a previous study involving 307 healthy children, 33% were found to carry *Staphylococcus aureus* on their tongues.[27] Nasal odor appeared intermittently in this study, with 8 subjects having nasal odor at only 1 appointment, 6 having nasal odor at 2 appointments, and only 1 subject with nasal odor that recurred at all 3 appointments (only 6 subjects had no nasal odor at all 3 appointments).

In a study examining the outcomes of functional endoscopic surgery in

children with chronic sinusitis,[7] among 52 patients (mean age 7.4 years), 47 (90%) and 33 (63%) children were considered to have purulent nasal discharge and postnasal drip, respectively. Thirty-five (67%) children were judged to have halitosis. After surgery 34 (66%) children reported that the malodor was resolved, and 5 (9%) children noted an improvement. However, because antibiotics were given after surgery, a direct association cannot be made between the success of surgery per se and improvement in bad breath. In a more recent investigation of 41 children with sinusitis,[8] foul breath was among the most common reports (N = 18).

As with the adult population, the role of tonsils in chronic malodor in children is unclear. Parents commonly report a transient oral malodor on the onset of sore throat in children.[12] Further investigations are necessary to assess the role of tonsils in pediatric oral malodor.

Among the various parameters studied, the Oratest scores proved the only measurement whose scores correlated with one another at all 3 appointments. When multiple regression analysis was carried out to test the contribution of plaque index and Oratest scores in the prediction of initial whole mouth malodor levels, both parameters contributed significantly ($P < .001$ and $P = .005$, respectively) to the regression equation, yielding a multiple r of 0.78. The data suggest that in children, plaque index and the Oratest measure largely independent microbial functions, both of which contribute to bad breath.

It is interesting that in contrast with previous data involving adult subjects,[24] the BANA test did not appear to provide significant associations with malodor parameters in the group of children studied. This result may be related to the detection limits of the test. Watson et al[26] found that although *T. denticola* and *P. gingivalis* were almost universally present in the plaque samples of children (as detected by enzyme-linked immunosorbent assay), their levels were not high enough to be detected by the BANA.

In summary, the results suggest that bad breath in children is related to both nasal and oral odor, with posterior tongue odor appearing to be very important. The types of odors encountered were similar to those encountered in adult subjects. Malodor parameters were generally improved, apparently by an increased awareness of the importance of oral hygiene. Because the results presented here are restricted to a selected population of children whose parents reported their having bad breath, it will be interesting to study the prevalence and nature of bad breath in the general pediatric population.

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50 Years Ago in The Journal of Pediatrics

BLOOD SUGAR IN NEWBORN INFANTS

Norval MA, Kennedy RLJ, Berkson J. J Pediatr 1949;34:542-51

Fifty years ago, Norval, Kennedy, and Berkson defined the normal concentration range of blood sugar (glucose) in term newborn infants as 15.0 to 120.0 mg/dL, with a mean of 61.0 ± 13.8 (SD) mg/dL; 95% of the infants had a blood sugar concentration between 32 and 70 mg/dL. Blood sugar increased 2.8 mg/dL per day over the first 6 days of life. Such values are perfectly acceptable and within the normal range today. Why are the blood glucose values that indicate hypoglycemia still controversial? First, many infants have transiently low glucose concentrations, but there are no data that directly link transient hypoglycemia to lasting neurologic impairment. Second, early surveys in preterm infants showed a statistically lower "normal" glucose concentration in the 20 to 40 mg/dL range. Third, rather inaccurate reagent strip methods of measurement of glucose concentration led many to accept a broader range of normal values in newborn infants, term or preterm.

Fortunately, several recent studies and management trends have reinstated the normal lower limit of glucose concentration in newborn infants, preterm or term, as above 50 mg/dL. First, more accurate and reliable bedside methods to rapidly measure blood glucose concentrations are now available. Second, a retrospective study by Lucas et al[1] showed increased incidences of mental and motor delays with increased numbers of episodes of low blood glucose concentration in preterm infants. There are risks associated with infants having repeated glucose concentrations below about 48 mg/dL. Third, a survey study by Srinivasan et al[2] provided mean and 95% CI values of plasma glucose concentrations in normal, healthy preterm infants, defining a lower 95% CI value of about 60 mg/dL by the second to third day of postnatal life. Finally, a study by Marconi et al[3] showed that healthy human fetuses from 20 weeks' gestation to term had umbilical venous glucose concentrations >50 mg/dL.

Clearly, there is no new evidence to support normal glucose concentrations in newborn infants, preterm or term, <50 mg/dL, confirming the observations of Norval, Kennedy, and Berkson from 50 years ago.

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Self-assessment of oral malodor 1 year following initial consultation

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Objective: *In an initial study, subjects complaining of bad breath were generally unable to score the level of their own oral malodor in an objective fashion. Subjects were taught several techniques for self-measurement of bad breath. One year following the initial consultation, subjects were recalled to determine whether their ability to assess their own oral malodor had improved.* **Method and materials:** *In the study, subjects were blinded to their own scores 1 year earlier, to the odor-judge scores, and to the results of the clinical laboratory tests. Thirty-two of 43 subjects in the original study who presented with a complaint of oral malodor agreed to participate in the follow-up study. Odor-judge scores and self-assessments of oral malodor (whole-mouth odor, tongue odor, and saliva odor) were compared with one another as well as with clinical parameters.* **Results:** *Objective improvements were noted in both oral health parameters and malodor levels of subjects. Despite this, self-assessments generally remained unrelated to objective parameters (odor-judge scores, clinical indices, and laboratory tests). Self-assessments were all significantly correlated with one another, and also were significantly associated with corresponding self-estimates made 1 year earlier.* **Conclusion:** *Subjects with a complaint of oral malodor remain largely unable to score their own bad breath in an objective fashion. In addition, they are not capable of sensing reductions in oral malodor 1 year following the original assessment, even though, from a clinical standpoint, improvements have taken place.* (Quintessence Int 1999;30:324–327)

Key words: halitosis, odor judge, oral malodor, self-assessment

CLINICAL RELEVANCE: Patients who complain of bad breath may harbor persistent subjective notions of how bad their breath is; these notions do not become objective over time, even when subjects have learned to smell their own oral odors and even when improvements have occurred.

Although it has been commonly written that people cannot smell their own oral malodor, this subject has rarely been investigated. In an initial study, 52 subjects (43 with a complaint of bad breath) were tested for their ability to score their own malodor, before, during, and after they were instructed in self-measurement techniques.[1] Subjects were taught to test themselves for whole-mouth odor by cupping their hands over the mouth. The subjects were also apprised that oral malodor is often reduced following proper dental treatment coupled with oral hygiene procedures, particularly flossing and tongue brushing.

One year following the initial consultation, 32 of the 43 subjects complaining of bad breath participated in the follow-up. The purpose of the present study was to detect changes in self-assessment 1 year following the initial testing and to compare these scores with changes in odor-judge scores, dental parameters, and laboratory tests.

METHOD AND MATERIALS

This single-center study was conducted in the dental clinics and Alpha Omega Research Laboratories at the Maurice and Gabriela Goldschleger School of Dental Medicine, Tel Aviv University, in central Israel.

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The probability of a significant difference among the 3 groups did not occur (Table 5). Nevertheless, examination of the curve in Fig 2, showing the variation of the plaque index from the beginning to the end of the test, reveals a clear tendency for the 2 CG groups to exhibit lower variations in the plaque index compared with the control group (without CG). This is in accordance with previous results,[8,9] which have shown that chewing gum can reduce the accumulation of plaque.

CONCLUSION

The limited number of subjects, in this exploratory study, necessitates careful conclusions. Nevertheless, it may be concluded that *(1)* chewing gum for 10 minutes did not modify the VSC, OS, and pH values recorded 3 hours later; *(2)* pH was more basic in the posterior part than in the anterior zone of the dorsum of the tongue, irrespective of time and presence or absence of chewing gum; *(3)* VSC scores rose transiently immediately after the test gum was chewed, and the organoleptic score fell in the first hour only after the test gum was chewed; and *(4)* chewing gum may have a valuable mechanical role in cleaning dental surfaces.

The results are ascribed to the masking effect of the 2 gums that were tested; a short masking effect appeared only in the test gum and may be the result of the chemical agent (menthol). Similar experiments should be considered with gums with other additive chemical agents exhibiting other properties, eg, antibacterial effects.

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Among the 43 subjects complaining of bad breath who participated in the original consultation, 32 agreed to participate in the present follow-up study. The subjects originally participating in the study were recruited following a radio interview and had a complaint of having bad breath.

Self-assessments

For self-assessments, subjects were asked to score their own oral malodor on a continuous 10-cm visual analogue scale that was marked as "no odor" on the 0-cm end, and as "extremely foul odor" on the 10-cm end.[1] Five self-assessments were made:

1. *Preconception score.* Prior to measurement, subjects were asked to score the level of bad breath that they thought they had at the time.
2. *Whole-mouth malodor score.* Subjects were instructed to smell the odor emanating from their entire mouth by cupping their hands over their mouth and nose while exhaling through the mouth and breathing in through the nose.
3. *Tongue malodor score.* Subjects were asked to extend their tongue and lick their wrist in a perpendicular fashion. Odor was judged by smelling the wrist after 5 seconds at a distance of about 3 cm.
4. *Saliva malodor scores.* Saliva samples (about 1 mL), obtained by expectoration following the dental examination, were allowed to stand in a closed Petri dish for 5 minutes at 37°C and then presented for odor assessment at a distance of about 4 cm.
5. *Postmeasurement score.* Immediately following the various self-assessments, subjects were again asked to rate their own oral malodor.

Subjects were blinded to all previous scores.

Organoleptic measurements

Organoleptic measurements of the whole mouth, tongue, and saliva were made by an odor judge (MR), whose scores have been shown to correlate with those of other judges as well as volatile sulfide levels.[2] Odor-judge assessments were carried out alongside the subject's self-measurements and scored analogously.

For whole-mouth odor, the subject was asked to breathe out from the mouth, at about 10 cm from the nose of the judge. Tongue and saliva odor were scored extraorally by the odor judge in the same manner as was employed for the subject's self-assessment, directly following each subject's appraisal. The odor judge was blinded to self-assessments as well as to all other parameters, including his own previous scores.

Clinical measurements

Dental parameters consisted of plaque and gingival indices as well as probing depth. Participants were scored for plaque accumulation and gingival health of all tooth surfaces (including wisdom teeth) with a plaque index (PI[3]) and a gingival index (GI[4]), respectively. Results presented represent the mean of all surfaces recorded. Mean probing depth measurements were recorded for all available sites.[5] Because odor levels of saliva are usually low, the saliva samples were obtained following the dental examination to potentiate the odor level.

RESULTS

Mean values of odor-judge assessments and self-assessments are presented in Table 1. The significance of overall reductions in scores, comparing follow-up to initial scores, was examined with a paired t test. Associations between initial and follow-up scores were sought with a linear regression analysis (Pearson).

Self-assessments

Self-assessment scores were, in all cases, lower than corresponding initial scores 1 year earlier, but differences did not, in most cases, reach significant levels. Overall, subjects did not sense a significant improvement either in whole-mouth odor or in saliva odor. Only in the case of tongue odor did subjects perceive a significant improvement over the status at the first consultation ($P = 0.017$). Follow-up self-assessment scores were significantly associated with corresponding initial scores. The highest associations were observed for tongue malodor ($r = 0.71$), followed by whole-mouth odor ($r = 0.63$) and postmeasurement ($r = 0.59$).

Table 2 summarizes follow-up correlations between preconception and postmeasurement self-estimates of whole-mouth, tongue, and saliva odor. All self-estimates were associated with one another, but the association between preconception and postmeasurement self-estimates was particularly high ($r = 0.81$; $P < 0.001$).

Organoleptic measurements

Corresponding odor-judge scores indicated that, whereas follow-up whole-mouth odor and saliva odor had significantly improved ($P = 0.006$ and $P = 0.009$, respectively), as compared with those initially measured, tongue odor had not changed overall ($P = 0.980$). The odor-judge scores at follow-up were significantly associated with initial scores, particularly with respect to tongue odor ($r = 0.491$; $P = 0.002$).

TABLE 1 Odor-judge assessments and self-assessments: Comparison with scores at initial consultation

Measurement	Mean ± SD	Significantly lower than at initial consultation*	Association with initial consultation scores†
Self-assessment			
Preconception	5.1 ± 2.7	$P = 0.303$	$r = 0.36$; $P = 0.022$
Whole-mouth odor	3.1 ± 2.4	$P = 0.222$	$r = 0.63$; $P < 0.001$
Tongue odor	5.0 ± 2.7	$P = 0.017$	$r = 0.71$; $P < 0.001$
Saliva odor	3.4 ± 2.8	$P = 0.495$	$r = 0.46$; $P = 0.004$
Postmeasurement	4.7 ± 3.1	$P = 0.016$	$r = 0.59$; $P < 0.001$
Odor-judge assessment			
Whole-mouth odor	3.3 ± 1.6	$P = 0.006$	$r = 0.32$; $P = 0.037$
Tongue odor	2.5 ± 1.5	$P = 0.980$	$r = 0.49$; $P = 0.002$
Saliva odor	2.4 ± 1.7	$P = 0.009$	$r = 0.42$; $P = 0.009$

* Paired *t* test.
† Pearson correlation.

TABLE 2 Pearson correlations comparing self-assessments at follow-up

	Whole-mouth	Tongue	Saliva	Preconception
Preconception	$r = 0.58$	$r = 0.65$	$r = 0.64$	—
	$P < 0.001$	$P < 0.001$	$P < 0.001$	
Postmeasurement	$r = 0.46$	$r = 0.67$	$r = 0.65$	$r = 0.81$
	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$

Clinical measurements

As in the initial study, odor-judge scores were generally associated with dental parameters, whereas self-assessment scores were not. In the first study, none of the self-assessments, with the exception of saliva scores, were associated with corresponding odor-judge scores. In the follow-up, there were no significant correlations between odor-judge scores and self-assessments whatsoever (Table 3).

With two exceptions (self-assessment of saliva versus GI: $P = 0.02$; tongue odor versus GI: $P = 0.05$), self-assessment scores were not associated with dental parameters. In contrast, odor-judge scores were, with one exception (whole-mouth odor versus PI: $P = 0.07$), significantly associated with dental parameters, eg, whole-mouth odor versus probing depth ($P = 0.006$).

Mean levels of PI, GI, and probing depth are summarized in Table 4. Whereas a significant reduction in GI was noted ($P = 0.025$), reductions in plaque index and probing depth were not significant ($P = 0.065$ and $P = 0.104$, respectively).

Among the 32 participants in the follow-up, 61% reported using floss, as opposed to 31% initially.

TABLE 3 Pearson correlation coefficients comparing odor-judge assessment and self-assessment at follow-up

Parameter	*r*	*P*
Whole-mouth odor	0.03	> 0.1
Tongue odor	0.15	> 0.1
Saliva odor	0.16	> 0.1

TABLE 4 Dental parameters at follow-up

Measurement	Mean ± SD	Significantly lower than initial scores*
Plaque index	1.0 ± 0.5 mm	$P = 0.065$
Gingival index	0.9 ± 0.4 mm	$P = 0.025$
Mean probing depth	2.6 ± 0.8 mm	$P = 0.104$

* Paired *t* test.

Following the initial consultation, 85% sought professional dental care. Only 35% thought that their bad breath had improved at the follow-up compared with initial levels.

DISCUSSION

It is generally acknowledged that people suffering from bad breath are often unaware of it, while many others perceive that they have terribly bad breath but, in fact, do not.[1] It was previously assumed that erroneous self-perception of oral malodor is due to adaptation, ie, dulling of the perception because of prolonged exposure.[6] In a previous study encompassing 52 subjects, 43 of whom complained of oral malodor, it was found that subjects were generally unable to score their own malodor in an objective fashion. However, no evidence was found to support a role for adaptation in the misguided scores. Rather, it was found that subjects harbor preconceptions of how bad their breath is and that subsequent self-measurements correspond to these subjective notions.[1]

In the present study, 32 of the 43 subjects with a complaint of malodor were retested 1 year following the original consultation. At the initial consultation, subjects were taught 3 ways of smelling their own oral malodor (cupping hands over mouth, licking the wrist and smelling, and smelling one's own saliva). They were also alerted to the association between dental health and oral malodor. Many (85%) sought professional dental services after the initial consultation. It is noteworthy that significant reductions in whole-mouth malodor, saliva malodor, and gingival index were evident 1 year following the initial consultation. Because none of the subjects were actually treated within the framework of the study, the data suggest that making subjects aware of the connection between oral malodor and oral health leads them to seek dental treatment, with subsequent improvements in malodor and periodontal disease–related parameters.

We predicted that, during the 1-year period, subjects would become increasingly adept and increasingly objective in assessing and scoring their own malodor. This, however, was not the case. No associations between odor-judge scores and self-measurements were found. Although odor-judge assessments indicated significant improvement in whole-mouth odor and saliva odor, subjects sensed no significant improvement. Indeed, on questioning, only 35% believed that any improvement had occurred. In contrast, subjects felt a significant improvement in tongue malodor, whereas odor-judge scores showed no improvement whatsoever (probably because the back of the tongue is very difficult to clean properly). Finally, although the subjects' postmeasurement scores were significantly lower than in the initial study, they remained high compared with the odor-judge scores and were closely associated with corresponding initial scores.

These data, taken together, suggest that, despite the initial consultation and instruction, subjects remained unable to score their own oral malodors in an objective fashion. Thus, in subjects with a complaint of oral malodor, self-estimation of the odor levels is largely determined by persistent, preconceived notions that do not become objective over experience and time. In contrast, in a recent study of 123 healthy young adults who were recruited by financial incentive rather than by complaint, some objectivity was apparent in their self-assessment scores of whole-mouth and tongue malodor.[7]

It thus appears that, in the general population, those who are not concerned about having bad breath do have some inherent, objective ability to determine its level. However, those who are concerned about the problem are largely subjective in their appraisal and are relatively unable to sense improvement. This constitutes a problem for clinics involved in diagnosis and treatment of bad breath.[8,9] Although objective reductions in oral malodor and dental indices have been observed in this and other studies, subjects often feel that the problem has not been alleviated. Indeed, in the present work, only 35% acknowledged that an improvement had taken place.

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Clinical dilemmas posed by patients with psychosomatic halitosis

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Patients affected by psychosomatic halitosis never wish to visit a psychologic specialist, because they cannot recognize their own psychosomatic condition. They also never doubt that they have offensive oral malodor. Other people's behavior, such as covering the nose or averting the face, is interpreted by these patients as an indication that their breath is offensive, and these behaviors or attitudes reinforce their belief that they have a strong oral malodor. To clarify whether the patient's perception of another individual's attitude is affected by his or her delusion, this article is focused on the relationship between the behavior toward oral malodor and the psychologic profiles of patients with psychosomatic halitosis. If a patient expects simple avoidance behavior from other individuals, the development of psychosomatic halitosis may be accelerated, as it becomes a self-fulfilling prophecy. Individuals who are concerned with their own oral malodor but exhibit no oral malodor may have latent psychosomatic tendencies and may be mentally immature. A protocol for referring a patient to a psychologic specialist is presented. (Quintessence Int 1999;30:328–333)

Key words: halitophobia, halitosis, psychosomatic dentistry, psychosomatic disease

CLINICAL RELEVANCE: This article describes the diagnosis and management of psychosomatic halitosis patients in general dental practice.

Since the biochemical pathogenesis of oral malodor has been elucidated,[1-23] simple treatment measures for halitosis have proven to be very effective in controlling oral malodor. Nevertheless, common treatments for halitosis, such as tongue cleaning, rinsing with mouthwash, and periodontal therapy, are perceived to be unsuccessful in patients who are extremely sensitive to and often delusional about other people's behavior toward their imagined oral malodor.

Among patients with psychosomatic halitosis, 2 subgroups are reported[13]: *(1)* patients who have "imaginary halitosis" without detectable oral malodor and *(2)* patients who suffer from oral malodor with accompanying psychosomatic tendencies. In the former case, a patient does not accept the fact that he or she has no offensive oral malodor. In the latter case, a patient may not believe that his or her oral malodor is reduced or eliminated after treatment and may continue to complain about the "halitosis" that does not exist any longer.

The patient's persistent complaints can frustrate clinicians. These patients interpret other people's behavior, such as covering the nose or averting the face, as an indication that their breath is offensive, and these behaviors or attitudes convince patients that they have a strong, offensive oral malodor.

Because psychosomatic conditions are caused by factors such as social phobia (fear of being around people), the patient may benefit from a referral to a psychologic specialist. However, patients usually refuse to visit a specialist, because they cannot recognize their condition as psychologic. They never doubt that they have an offensive oral malodor. Such patients can be quite unhappy with a dentist who cannot detect their malodor and who suggests a referral for psychologic evaluation.

Although many discussions of psychogenic halitosis have been published since 1974,[24-30] a successful protocol for referring patients to a psychologic specialist has not yet been reported. In this article, the background of psychosomatic halitosis will be discussed. Also, suggestions for facilitating the referral of a patient with psychosomatic halitosis to a specialist will be made.

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